Universidade de Aveiro Departamento de Biologia
2008

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Prof. Doutor Rui Medeiros, Professor Auxiliar Convidado do Departamento de Patologia e Imunologia Molecular do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto, e co-orientação científica da Profª. Doutora Sónia Mendo, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

O JÚRI

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Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Profª Doutora Sandra Maria Araújo da Costa

Post-Doctoral Fellow, Life and Health Sciences Research Institute, Health Science School, Minho University

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RESUMO

Introdução: Os factores de crescimento desempenham um papel fundamental na regulação da proliferação celular, e o seu descontrolo é uma característica do desenvolvimento maligno. O gene *EGF* codifica um factor de crescimento que se liga ao receptor EGFR envolvido na activação das vias que promovem a proliferação, sobrevivência, migração e diferenciação celular, através da estimulação de uma cascata de cinases. Estudos recentes propõem que a variabilidade genética, como os SNPs, poderá desempenhar um papel significativo na predisposição para cancro. Objectivos: Neste estudo avaliou-se a correlação do polimorfismo do gene *EGF*, na posição 61 (A/G), e a susceptibilidade para cancro do ovário, mama e colo do útero.

Material e Métodos: Para tal, procedeu-se à análise por PCR-RFLP, de 1442 amostras de DNA, 175 de indivíduos com carcinoma do ovário, 383 com caricinoma da mama, 384 com lesões cervicais e 500 mulheres sem doença oncológica.

Resultados: Concluiu-se, neste estudo, que os indivíduos portadores do alelo G e com carcinoma do ovário tem menor risco de desenvolver esta neoplasia (OR=0,72; 95%CI: 0,55-0,94; p=0,012). Esta protecção também ocorre em mulheres com idade inferior a 53 anos (OR=0,63; 95%CI: 0,43-0,91; p=0,009), assim como para doença avançada (OR=0,63; 95%CI: 0,45-0,89; p=0,006). No tempo de aparecimento de doença, os portadores do genótipo GG apresentam este cancro mais tarde que os portadores do alelo AA (p=0.035). O mesmo efeito é observado no cancro da mama, observa-se menos risco para desenvolver a doença (OR=0,82; 95%CI: 0,08-1,00; p=0,012), assim como os portadores GG desenvolvem a doença mais tarde (p=0,041). As mulheres mais jovens, com cancro cervical em doença avançada e portadoras de G têm maior risco de desenvolver esta neoplasia (OR=3,17; 95%CI: 1,21-8,26; p=0,016).

Discussão: Com base nestes resultados, pode inferir-se que a presença do alelo G poderá estar associada a um efeito protector para desenvolvimento de cancro do ovário e da mama. Estes carcinomas apresentam um aumento significativo de expressão de EGFR, e a protecção poderá ser explicada pelo facto dos portadores do alelo G terem maior produção de EGF, e de este estar envolvido na internalização e degradação do seu receptor e consequentemente ocorrer menor expressão de EGFR à superfície da célula.

No carcinoma cervical um aumento do factor de crescimento poderá aumentar a sinalização das vias activadas por EGF por parte dos portadores G.

ABSTRACT

Introduction: Growth factors perform an essential role in the cellular proliferation regulation and its miss control is a characteristic of malignant development. The EGF gene codifies a growth factor that binds to the EGF receptor involved in the activation of ways that promote the proliferation, survival, migration and cellular differentiation, through the stimulation of a kinase cascade. Recent studies propose that the genetic variability, as the SNPs, may perform a significant role in the cancer predisposition. This study had the objective of appraising the correlation of *EGF* gene A61G polymorphism and the susceptibility of ovarian, breast and cervical cancer.

Material and methods: In this manner we proceeded to the analysis of 1442 DNA sample by PCR-RFLP, 175 people with ovarian cancer, 383 with breast cancer, 384 with cervical lesions and 500 women without cancer.

Results: We concluded that G carriers with ovarian cancer had less risk of developing this neoplasia (OR=0,72; 95%CI: 0,55-0,94; p=0,012). This protection also occurs in women with ages below 53 years (OR=0,63; 95%CI: 0,43-0,91; p=0,009), as well advanced disease (OR=0,63; 95%CI: 0,45-0,89; p=0,006). In the time to onset disease analysis, GG genotype subjects present this cancer later than AA carriers (p=0.035). The same effect is observed in breast cancer with less risk to develop this disease (OR=0,82; 95%CI: 0,08-1,00; p=0,012) and the GG carriers develop this disease later (p=0,041). The younger women G carriers with advanced cervical cancer have more risk to develop this disease (OR=3,17; 95%CI: 1,21-8,26; p=0,016).

Discussion: With support in these results, we can deduce that the presence of G carrier may be associated with the protection effect of ovarian and breast cancer development. These cancers show a significative increase of EGFR expression and this protection can be explained by the fact that G carrier subjects have a higher production of EGF and this one is involved in the internalization and degradation of its receptor and consequently occurring less EGFR expression at the cell surface.

In cervical cancer an increase of this growth factor may increase the pathways activated to EGF in G carriers.

Índice

1. INTRODUÇÃO

1. INTRODUÇÃO

Todos os cancros são causados por anomalias na sequência do DNA. Ao longo da vida, o DNA das células humanas está exposto a agentes mutagénicos, assim como à ocorrência de erros na sua replicação, resultando em progressivas e subtis mudanças na sequência de DNA de cada célula [Futreal *et al.,* 2001]. Ocasionalmente, mutações somáticas alteram a função de genes críticos, como proto-oncogenes ou supressores tumorais, que favorecem a promoção do crescimento [Futreal *et al.,* 2001; Hoeijmakers, 2001]. Contudo, as mutações espontâneas são insuficientes para explicar o risco para cancro ao longo da vida [Hoeijmakers, 2001].

A etiopatogenia do cancro está relacionada com eventos epigenéticos e interacções celulares. As neoplasias malignas dependem de factores intrínsecos ao genoma humano, sendo aceite o papel de mutações autossómicas em genes que codificam moléculas com funções no crescimento tumoral, na regulação da apoptose e na reparação do DNA [Ponder, 2001]. Contudo, estas mutações genéticas não explicam completamente o complexo processo patofisiológico de formação e desenvolvimento das células tumorais, sugerindo que outros factores genéticos, nomeadamente polimorfismos com repercussão funcional na molécula que codificam, possam ter um papel relevante em oncobiologia. Variações genéticas comuns determinam uma susceptibilidade individual para cancro, bem como risco para doença avançada, sugerindo que, para além de um papel activo na oncogénese, influenciam a progressão e facilitam a metastização à distância [Ponder, 2001]. Mais recentemente, foi demonstrada a sua importância na resposta a terapêutica oncológica, ou seja, a farmacogenómica [Silva, 2005]. Estas novas pesquisas fornecem informação que permite desenhar estratégias de prevenção nos grupos de alto risco [Ponder, 2001].

1.1 CANCRO

Neoplasia significa "novo crescimento", termo igualmente designado por tumor. Um neoplasma é uma massa anormal de tecido, com crescimento excessivo, descontrolado e anormalmente rápido, mesmo após a interrupção dos estímulos que deram origem à mudança [Kumar *et al.*, 2003]. Os tumores são constituídos por células transformadas, as células neoplásicas, e por células não transformadas, constituindo estas, o estroma de sustento, como vasos sanguíneos e tecido conjuntivo [Robbins *et al.*, 1999]. O microambiente envolto no tumor pode ter uma participação activa na etiologia, progressão e metastização do cancro [Liotta *et al.*, 2001]. Células do estroma e tumorais trocam enzimas e citocinas que podem modificar o local da matrix extracelular, estimulando a migração, e promovendo a proliferação e sobrevivência celular [Liotta *et al.*, 2001].

Cancro é o termo comum utilizado para designar os tumores malignos. Uma célula cancerígena pode ser caracterizada por três tipos de modificações: imortalização (capacidade de multiplicação indefinida); transformação (independência de factores de controlo); capacidade de invasão e metastização (invasão tecidos normais) [Lewin, 2000].

A distinção entre tumores benignos e malignos assenta na sua morfologia e evolução clínica. Nos primeiros, as células assemelham-se às normais, crescem como massas coesivas desenvolvendo uma cápsula na periferia, não invadindo os tecidos circundantes. Nos tumores malignos, o crescimento é geralmente mais rápido e as células que os constituem distinguem-se das células normais quanto ao grau de diferenciação (podendo variar desde grau bem diferenciado até pouco diferenciado). As células bem diferenciadas assemelham-se às células normais, enquanto as células pouco diferenciadas apresentam baixo nível de especialização, formando tecidos anaplásicos. Estes caracterizam-se pela variação da forma, assim como pelo aumento do tamanho das células e respectivos núcleos, apresentando desorganização total da arquitectura tecidular [Robbins *et al.*, 1999; Kumar *et al.*, 2003].

As neoplasias malignas são precedidas por um crescimento desordenado não neoplásico (displasia). Quando as alterações displásicas são acentuadas afectando a espessura do epitélio, a lesão é designada como neoplasia pré-invasiva (carcinoma *in situ*). Após invasão, as células neoplásicas malignas penetram nos tecidos circundantes,

destruindo-os. Quando a invasão atinge cavidades corporais, vasos sanguíneos e linfáticos, ocorre uma dispersão originando crescimento secundário noutros locais, formando-se metástases [Robbins *et al*., 1999].

Os tumores malignos podem dividir-se em carcinomas e sarcomas. Os primeiros têm origem epitelial e subdividem-se histologicamente em vários tipos, como adenocarcinomas (grandulares) e carcinomas de células escamosas. Os sarcomas originam-se a partir de tecidos mesenquimatosos [Kumar *et al*., 2003].

1.1.1 EPIDEMIOLOGIA

A probabilidade de um indivíduo vir a desenvolver cancro está relacionada com as taxas de incidência e de mortalidade [Kumar *et al.*, 2003]. Estas taxas referem-se, respectivamente, ao número de novos casos e ao número de óbitos que ocorrem, sendo expressas num valor absoluto de casos por ano ou número de casos por l00 000 habitantes por ano. A prevalência descreve o número de pessoas vivas com a respectiva doença [Parkin *et al.*, 2005].

Foi estimado que, em todo o mundo, no ano 2000, surgiram 10 milhões de novos casos, 6 milhões de mortes por cancro e 22 milhões de pessoas vivas com cancro [Parkin *et al.*, 2001]. A nível mundial, o cancro do pulmão é o mais frequente no sexo masculino (902 000 novos casos no ano 2000) enquanto no feminino o mais frequente é o da mama (1 050 000 novos casos no ano 2000) (Fig1). Um segundo grupo de tumores mais frequentes inclui cancro do colo do útero, colorectal e gástrico nas mulheres e o prostático, colorectal e gástrico nos homens [Shibuya *et al.*, 2002]. O cancro com maior prevalência é o da mama, sendo também o que apresenta uma sobrevida mais elevada (dados de 2002 indicaram 4,4 milhões de sobreviventes num período de 5 anos após diagnóstico) [Parkin *et al.*, 2005].

Em Portugal são diagnosticados 40 a 45 mil novos casos de cancro por ano, valores similares a vários outros países da União Europeia. No entanto, enquanto nestes países a mortalidade tem vindo diminuir, em Portugal, pelo contrário, continua a aumentar a uma taxa anual de 6%. O cancro é a segunda causa de morte no nosso país (a taxa de mortalidade no ano 2001 foi de 213,2 por 100.000 habitantes). Em Portugal, os tumores malignos mais frequentes no sexo masculino são o colorectal, prostático, gástrico e do

pulmão. Nas mulheres, são as neoplasias malignas da mama, colorectal, gástrico e do útero [Simões *et al.*] (Fig2).

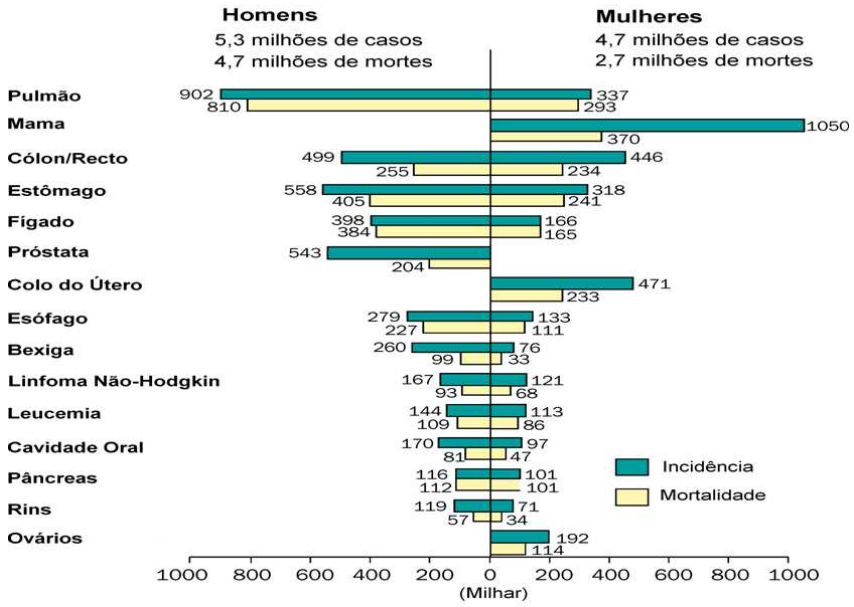

Figura 1. Incidência de novos casos de cancro e mortalidade mundiais, no ano 2000, em função do sexo. (Adaptado de [Parkin, 2001]

A incidência de cancro varia consideravelmente ao longo das décadas, devido a factores ambientais, mudanças culturais, alteração de hábitos nutricionais, emigração, entre outros. Em Portugal, os dados revelam um aumento na taxa de mortalidade no sexo masculino e um decréscimo no sexo feminino [Pinheiro *et al.*, 2003].

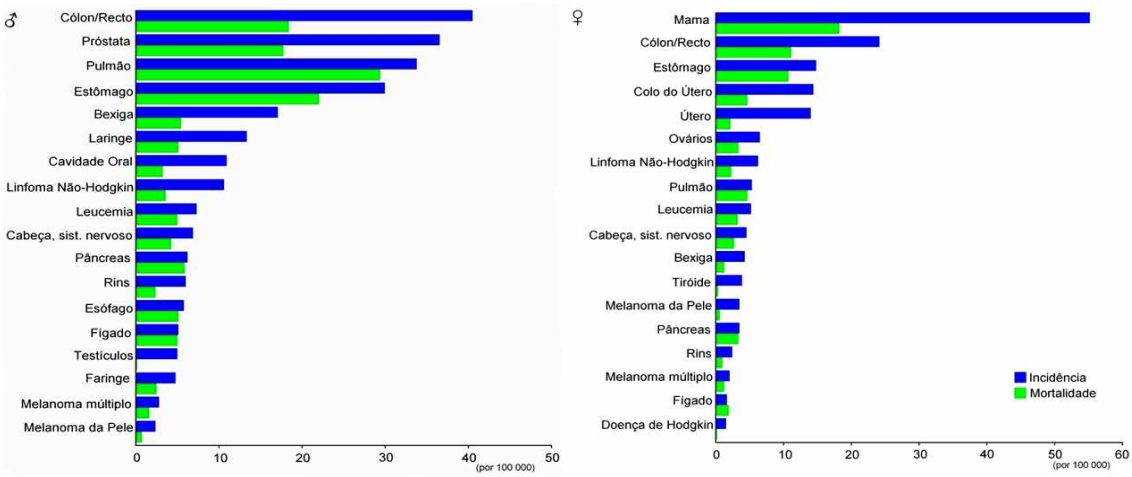

Figura 2. Incidência de novos casos de cancro e mortalidade mundiais, no ano 2000, em função do sexo. (Adaptado de [Simões *et al.*]

Nos homens este aumento poderá estar associado, entre outros factores, ao aumento da incidência de cancro do pulmão; a diminuição de incidência observada nas mulheres poderá estar associada ao decréscimo de óbitos por cancro no colo do útero, uma das neoplasias mais comummente diagnosticadas neste sexo [Kumar *et al.*, 2003].

O risco de um indivíduo poder vir a desenvolver cancro encontra-se igualmente correlacionado com a idade. Em idades muito avançadas a tendência para desenvolver cancro invasivo pode atingir 1 em cada 2 homens e 1 em cada 3 mulheres [DePinho, 2000]. Com o aumento da idade ocorre uma perda da manutenção do genoma, sucedendo acumulação de mutações, alterações na regulação epigenética, disfunção dos telómeros e alterações no microambiente envolta da célula, que conduz a uma maior senescência celular [DePinho, 2000; Finkel *et al.*, 2007; Garinis *et al.*, 2008].

1.1.2 Biologia Molecular

O cancro é hoje compreendido como uma doença genética que pode ser adquirida nas células somáticas ou herdada na linha germinativa [Robbins *et al*., 1999; Griffiths *et al.*, 2000].

O tumor origina-se a partir de uma só célula que sofreu lesão genética, com potencial de crescimento celular [Kumar *et al.*, 2003]. A progressão tumoral é adquirida de modo gradual, como resultado da acumulação de mutações sucessivas [Klein *et al*., 1985; Robbins *et al*., 1999]. Os principais alvos desta lesão genética são genes reguladores comuns, pertencentes a quatro classes: *proto-oncogenes, genes supressores tumorais, genes que regulam a apoptose e genes envolvidos na reparação do DNA* [Robbins *et al*., 1999].

1.1.2.1 Crescimento Celular e Morte Celular Programada

O desenvolvimento tumoral não resulta necessariamente dum aumento da proliferação celular. Resulta, antes sim, de um desequilíbrio entre progressão do ciclo celular (divisão celular) e crescimento celular (massa de células) por um lado, e morte celular programada por outro [Blume-Jensen *et al.*, 2001].

O ciclo celular é controlado de modo a que os eventos ocorram de forma sequencial. Na sua progressão intervêm ciclinas e cinases dependentes de ciclina (CDKs), que actuam através de um processo de fosforilação/desfosforilação em proteínas alvo. A fosforilação de proteínas alvo inicia uma cadeia de eventos que culmina na activação de determinados factores de transcrição. Um dos alvos é o gene do retinobastoma *(Rb)*, que se encontra associado ao factor de transcrição E2F, e cuja fosforilação induz a libertação de E2F, promovendo-se o início da fase S do ciclo celular [Griffiths *et al*., 2000; Evan *et al*., 2001; Kumar *et al*., 2003].

A morte celular programada, ou apoptose, elimina células anormais com potencial para causar danos. Após activação duma cascata de enzimas (caspases), ocorre a destruição de vários sistemas estruturais e funcionais dentro da célula. Inicialmente, há uma fragmentação do DNA dos cromossomas, rompimento da estrutura organelar e perda da forma normal da célula. Num momento posterior, as células fragmentam-se, formando os corpos apoptóticos, que são fagocitados [Griffiths *et al*., 2000; Evan *et al*., 2001].

O balanço entre proliferação celular e apoptose encontra-se controlado por complexas vias de sinalização que se ajustam às condições envolventes. Deste modo, há sinais de natureza intra ou extra-celular que estimulam ou inibem o crescimento, ou a apoptose [Griffiths *et al*., 2000] (Fig3).

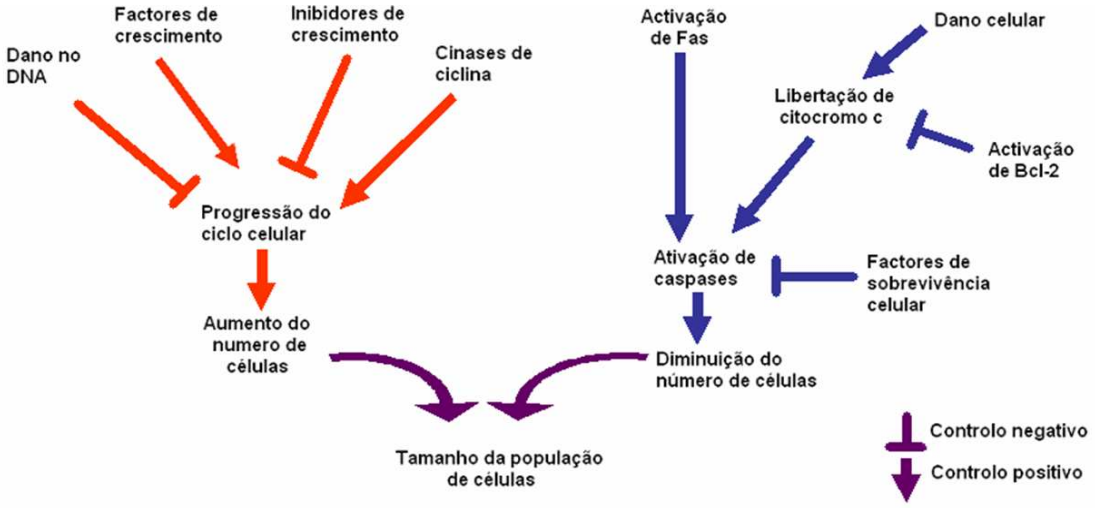

Figura 3. Mecanismo de controlo da proliferação celular. (Adaptado de [Griffiths *et al.*, 2000]

Sinais intracelulares- Para permitir a reparação de lesões no DNA, a proteína tumoral p53, pode induzir a paragem do ciclo celular por inibição indirecta de CDKs, exercendo um controlo negativo, sob o ciclo celular. Se a reparação não for possível induz apoptose,

exercendo um controlo positivo [Lewin, 2000; Evan *et al*., 2001; Ponder, 2001; Kumar *et al*., 2003]. A libertação do citocromo c mitocondrial para o citoplasma promove a activação de caspases que activam a apoptose [Lewin, 2000; Evan *et al*., 2001]. Pode ocorrer também um controlo negativo da apoptose, através da proteína CLL/Linfoma 2 de Celula B (BCL-2), impedindo que o citocromo c se liberte [Griffiths *et al.*, 2000; Evan *et al*., 2001; Kumar *et al*., 2003].

Sinais extracelulares- Os sinais extracelulares controlam o tempo de vida e a proliferação das células de um tecido, órgão ou organismo. Neste controlo, existe comunicação entre células especializadas que libertam ligandos, sob a forma de sinais endócrinos libertados na circulação sanguínea, ou parácrinos que apenas actuam localmente. Estes ligandos actuam na transdução de sinal para a divisão celular. Ligam-se a proteínas receptoras transmembranares, que iniciam sinais químicos no citoplasma através de moléculas intermediárias, que por sua vez activam ou reprimem factores de transcrição [Griffiths *et al.*, 2000].

Nos sinais extracelulares que controlam a apoptose intervém o sistema Fas (via associada ao domínio de morte). Uma célula adjacente possuindo o ligando Fas (exposto na membrana celular) liga-se ao receptor Fas da superfície da célula alvo, e este complexo activa o factor de activação da protease apoptótica, Apaf, que por sua vez induz a cascata de caspases [Griffiths *et al*., 2000].

1.1.2.2 Carcinogénese

Um tumor maligno resulta da acumulação de alterações específicas na célula, que incidem nos processos de estimulação da proliferação ou inibição da apoptose [Griffiths *et al*., 2000; Evan *et al*., 2001]. O fenótipo maligno é determinado por sete alterações que modificam funções celulares essenciais: autosuficiência relativamente aos sinais de estimulação do crescimento; insensibilidade aos sinais inibidores do crescimento; perda de mecanismos de apoptose; defeitos na reparação do DNA; potencial infinito de replicação; angiogénese; capacidade de invasão e mestatização [Kumar *et al.*, 2003].

De acordo com o supra referido, estas alterações ficam a dever-se essencialmente à ocorrência de mutações em conjuntos de genes que são agrupados segundo as suas funções:

Oncogenes: constituem proto-oncogenes que sofreram modificação e que adquiriram a capacidade de promover o crescimento celular na ausência de factores de crescimento. Desta forma, a transformação de um proto-oncogene em oncogene constitui um ganho de função, as proteínas por eles codificadas, são activadas, e o gene alterado será dominante [Lewin, 2000; Ponder, 2001]. Os oncogenes podem codificar factores de crescimento, receptores de factores de crescimento, proteínas de transdução de sinal, factores de transcrição, ciclinas e cinases dependentes de ciclinas [Lewin, 2000; Ponder, 2001; Kurnar *et al*., 2003].

Genes de supressão tumoral: como os genes *Rb* e o *p53*, regulam o crescimento celular impedindo a formação de tumores a partir de células normais. De acordo com a hipótese de Knudson, a alteração nos dois alelos destes genes resulta na perda da sua função, promovendo a formação de tumor [Lewin, 2000; Ponder, 2001; Kurnar *et al.*, 2003] .

Genes reguladores da apoptose: as mutações ocorridas nestes genes podem ter um papel no desenvolvimento de neoplasias. Por um lado, ao aumentarem o tempo de vida da célula, possibilitam a acumulação de mutações promotoras de proliferação e, por outro, permitem a multiplicação de células com mutações tumorais (por exemplo o gene *BCL-2*) [Griffiths *et al.*, 2000; Kumar *et al.*, 2003].

Genes de reparação de DNA: codificam proteínas reparadoras de DNA, quando este é alvo de uma agressão, como agentes nocivos tais como radiação ionizante, luz solar, carcinogéneos ambientais, stresse oxidativo gerado pelo metabolismo; ou quando ocorrem erros na replicação do DNA [Friedberg, 2003; Kumar *et al.*, 2003]. Alterações neste grupo de genes permitem a ocorrência de mutações, nomeadamente em genes directamente relacionados com tumores. A importância destes genes pode ser observada nos cancros hereditários da mama nas famílias com diversos membros afectados, em que 80% contêm mutações nos genes *BRCAl* e *BRCA2,* dado estar envolvido no processo de recombinação homóloga e reparação do DNA [Kumar *et al.*, 2003].

1.1.2.3 Bases Moleculares da Progressão Tumoral

Para ocorrer tumorigénese são necessárias diversas alterações genéticas sequenciais ou simultâneas. Para uma transformação completa é necessária cooperação, uma vez que cada oncogene tem a capacidade de induzir parte do fenótipo tumoral. Nas células cancerígenas

estão inactivos pelo menos dois genes supressores tumorais, encontrando-se activos diversos oncogenes [Kumar *et al.*, 2003]. Estima-se que são necessários entre 4 a 7 eventos genéticos para desenvolver a carcinogénese [Ponder, 2001]. Estas alterações encontram-se em diversas vias envolvidas na carcinogénese, divergindo quer para os diferentes tipos de cancro, quer dentro do mesmo tipo [Ponder, 2001]. Os oncogenes e genes supressores tumorais regulam directamente a entrada da célula nas vias tumorigénicas.

Existe outro grupo de genes que não controlam directamente o crescimento tumoral mas que afectam a estabilidade genómica. Dentro desta categoria temos os genes reparadores de DNA, que promovem um aumento de mutações em todos os genes, incluindo os que estão directamente relacionados com o crescimento tumoral [Kumar *et al.*, 2003].

A maioria parte dos tumores tornam-se mais agressivos com o tempo, em parte devido à maior capacidade de invasão e metastização. Esta progressão resulta da acumulação de mutações múltiplas (devido à perda de função da *p53* ou de genes que regulam a reparação de DNA), assim como do microambiente envolvente que contribui com a produção de moléculas promotoras do crescimento tumoral [Kumar *et al.*, 2003].

1.1.2.4 Factores de Susceptibilidade

Existe um conjunto de factores que aumentam a susceptibilidade de desenvolvimento de cancro, podendo ser agrupados nas seguintes classes:

Substâncias químicas: Determinadas substâncias químicas produzem transformação neoplásica através de 2 passos: iniciação (indução de mutações irreversíveis) e promoção (indução dum tumor). A sua acção pode ser directa, sem alteração química no metabolismo, ou indirecta, quando a substância tem acção carcinogénica apenas devido a uma alteração metabólica. Estes agentes activos são altamente reactivos, sendo o DNA o seu alvo primário para o desenvolvimento de neoplasias [Kumar *et al.*, 2003]. Devido à exposição a químicos, determinados ambientes industriais estão associados ao aumento da ocorrência de cancro.

O cancro do pulmão é um exemplo clássico de neoplasia associada a substâncias químicas, uma vez que a sua incidência é mais elevada em fumadores do que em não fumadores, em particular naqueles que iniciaram o hábito muito jovens, não o havendo interrompido durante décadas. O efeito carcinogénico do tabaco encontra-se igualmente

relacionado com cancro do pâncreas, bexiga, rins, laringe, boca, faringe, esófago, estômago, fígado e, provavelmente, do colo do útero [Peto *et al.*, 2001].

Dieta e obesidade: Alguns nutrientes da dieta como álcool, determinados alimentos (alguns associados a costumes locais como enchidos, salgados, etc.) e a presença de anflotoxinas têm uma forte relação com cancro. A obesidade, normalmente associada a hábitos alimentares, está igualmente relacionada com a incidência de cancro da mama e endométrio, após a menopausa [Peto *et al.*, 2001].

Factores hormonais e reprodutivos: Hormonas endógenas ou exógenas estão associadas a risco aumentado de cancro de ovário e mama. No caso do cancro da mama, a administração de contraceptivos orais ou terapia por reposição hormonal parece aumentar a susceptibilidade para esse tipo de tumor. Por outro lado, a incidência de carcinoma mamário é menor por menarca tardia, menopausa prematura, paridade precoce e/ou alta paridade [Peto *et al.*, 2001].

Virus e Bactérias: Algumas formas de tumores são de origem viral [Kumar *et al.*, 2003]. A actividade transformante de um vírus tumoral depende de um ou mais genes virais e diz respeito à alteração das propriedades de multiplicação da célula alvo através da modificação da sua estrutura reguladora [Lewin, 2000]. O papilomavirus humano (HPV), sexualmente transmissível, está presente em todos os carcinomas cervicais uterinos, nomeadamente os tipos 16, 18, e 45 [Peto *et al.*, 2001]. Outros vírus, foram também associados a diferentes tipos de cancro; é o caso do vírus da hepatite B (HBV) com o cancro do fígado; do vírus da hepatite C (HCV) com o cancro do estômago, fígado, ou colo do útero; bem como o vírus de Epstein-Barr (EBV) com linfomas de células B ou carcinomas da nasofaringe [Peto *et al.*, 2001; Kumar *et al.*, 2003].

Helicobacter pylori, uma bactéria que provoca infecção gástrica crónica, pode ser também um co-factor de risco importante para cancro do estômago [Peto *et al.*, 2001].

Factores físicos: A exposição a radiação ionizante, luz ultravioleta e amianto aumentam o risco de cancro [DeVita *et al.*, 2001].

1.1.2.5 Desregulação Genética

A instabilidade genómica presente nas células cancerígenas resulta de mutações herdadas ou adquiridas nas células somáticas [Balmain *et al.*, 2003]. Estas alterações genéticas podem ocorrer a vários níveis, como alterações cromossómicas, amplificações de

genes e metilações [Balmain *et al.*, 2003; Kumar *et al.*, 2003]. As alterações cromossómicas estão associadas a fenómenos tardios da progressão de cancro e podem incidir no número de cromossomas (aneuploidia) ou na sua reorganização (translocações e inversões). A amplificação de proto-oncogenes pode ocorrer centenas de vezes no seu genoma, conduzindo a uma sobre-expressão dos seus produtos. A hipermetilação de promotores associa-se de igual forma à tumorigénese, devido ao silenciamento de genes supressores [Kumar *et al.*, 2003].

A predisposição para cancro pode ocorrer através da presença de alelos raros mas altamente penetrantes, estes aumentam fortemente o risco de cancro quando herdados pela linha germinativa [Balmain *et al.*, 2003]. Esta forte predisposição está associada a genes responsáveis pela carcinogénese, tais como alterações nos processos de reparação de DNA ou na estabilidade genómica [Ponder, 2001]. Incluem-se neste grupo o retinoblastoma, polipose adenomatosa familiar, e mutações nos genes *BRCAl* e *BRCA2* [Peto *et al.*, 2001; Balmain *et al.*, 2003].

A predisposição para cancro por combinação de variações genéticas pouco penetrantes pode ter ainda maior significado para a saúde pública quando comparadas com alelos raros muito penetrantes e herdados [Balmain *et al.*, 2003]. Este argumento é suportado por dados relativos ao cancro da mama, em que dos cancros associados a risco familiar (5 a 10%) apenas 15 a 20% são atribuídas a mutações nos genes *BRCAl* e *BRCA2* [Ponder, 2001; Balmain *et al.*, 2003]. Os restantes 80 a 85% têm origem genética, ambiental ou mista, embora possa existir uma predominância de factores genéticos [Balmain *et al.*, 2003]. Neste grupo coexistem um pequeno número de genes muito penetrantes e um grande número de genes pouco penetrantes. Estes, incluem genes que codificam receptores hormonais, factores de crescimento, genes envolvidos no metabolismo de xenobióticos e reparação de DNA. A variação dos seus alelos pouco penetrantes encontra-se associado a um risco de aproximadamente 150%. Variações particulares estão associadas à susceptibilidade para doenças, ou permitem avaliar a sobrevivência e a resposta a tratamentos. A maior parte destas variações são polimorfismos de um único nucleótido (SNPs), existindo milhões no genoma humano, encontrando-se descritos em bases genómicas [Ponder, 2001].

1.1.3 TRANSDUÇÃO DE SINAL

A regulação genética da célula está dependente da percepção, através de proteínas transmembranares, de sinais vindos do exterior. Estas proteínas podem constituir canais iónicos, transportadores ou receptores. Para ocorrer transmissão de um sinal, um ligando extracelular interage com uma proteína transmembranar, produzindo uma actividade no domínio intracelular da membrana. Ocorre uma transdução de sinal, capaz de provocar uma actividade catalítica no citosol, permitindo a amplificação do sinal exterior à célula [Lewin, 2000].

No grupo de receptores membranares encontram-se as proteínas cinase, às quais se ligam citocinas, como o factor de crescimento epidérmico, EGF, ou o factor de crescimento derivado de plaquetas, PDGF, insulina e muitos outros. Os receptores podem actuar de diversas formas. Alguns receptores são de tirosina-cinase (RTK), outros serina-treonina cinase. A sua actividade básica é adicionar um grupo fosfato a uma proteína alvo. A actividade cinásica encontra-se igualmente em proteínas livres no citosol, também elas envolvidas na transdução de sinal [Lewin, 2000]. Existem de igual forma receptores sem actividade enzimática, cuja mudança conformacional após a ligação do ligando altera a conformação das proteínas citoplasmáticas [Griffiths *et al.*, 2000].

Nos RTK, o seu domínio tirosina-cinásico é o elemento chave para o funcionamento dos receptores de factores de crescimento. Os RTK podem activar mensageiros secundários (que actuam numa via, activando ou inibindo uma proteína alvo) ou uma via efectora em cascata, normalmente proteínas cinase [Lewin, 2000]. Quando o ligando e o RTK se ligam, ocorre a dimerização de dois receptores e sua autofosforilação (a cinase do receptor fosforila as suas tirosinas do domínio citoplasmático), iniciando-se uma cascata de transdução de sinal [Griffiths *et al.*, 2000]. Os receptores EGF ou PDGF são do tipo RTK, responsáveis pela activação da proteina Ras [Lewin, 2000] (Fig4).

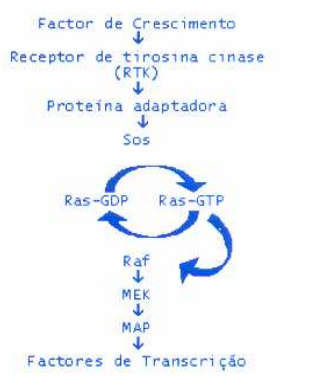

Figura 4. Via de sinalização de RTK, Ras, MAPK, envolvidas na transdução de sinal. (Adaptado de [Griffiths *et al.*, 2000]

O Ras, pertence à família de proteínas G e a sua actividade depende da sua ligação a guanina trifosfato (GTP) (estado activo) ou a guanina difosfato (GDP) (estado inactivo). Para activar Ras, o receptor associa-se à proteína adaptadora, receptor de factor de crescimento – proteína fronteira 2, Grb2 (*Growth Factor Receptor-Bound Protein 2*), sem fosforilação, que apenas se liga a SOS (*Son of Sevenless*), sendo suficiente para activar Ras [Lewin, 2000].

A activação de Ras conduz à activação duma cascata de proteínas através de fosforilações consecutivas, até à fosforilação de factores de transcrição. Inicialmente, a Ras activa a Raf, que activa a cinase MEK, que por sua vez activa a proteína activada por mitogénio-cinase, MAP-cinase (*Mitogen-Activated Protein*), designando-se a via proteína cinase activada por mitogénio, MAPK (*Mitogen-Activated ProteinKinase*). MAP-cinase move-se para o núcleo, onde fosforila factores de transcrição (como o Myc), que associam-se a genes para activar as vias da multiplicação ou direnciação celular [Lewin, 2000]. Os factores de transcrição ligam-se ao DNA em locais específicos, através de um conjunto determinado de aminoácidos (a.a.) [Kumar *et al.*, 2003], desencadeando alterações fenotípicas que vão desde a multiplicação até à diferenciação, conforme o tipo de células [Lewin, 2000].

A célula possui outras vias alternativas que podem substituir as supra referenciadas. Como exemplo, receptores serpentina também podem activar a via MAPK. Existem, também, várias vias de sinalização MAPK, com componentes análogos, em que cada um funciona de modo linear, podendo ocorrer comunicação cruzada entre as vias. Curiosamente, a duração do estímulo na via MAPK e outras, pode constituir um parâmetro

crítico, já que uma activação persistente pode levar à diferenciação, enquanto uma activação transitória à proliferação [Lewin, 2000].

1.1.3.1 EGFR- Receptor do Factor de Crescimento Epidérmico

A subclasse I de receptores da superfamilia tirosona cinase (RTK) é formada por ErbB (*V-ERB-B2 Avian Erythroblastic Leukemia Viral Oncogene*) ou chamados receptores do factor de crescimento epidérmico (EGF), e inclui 4 membros: EGFR (também chamado ErbB1/HER1), ErbB2 (Neu/Her2), ErbB3 (HER3) e ErbB4 (HER4). O gene humano é designado *ErbB* devido à decoberta do oncogene *v-erbB* de *avian erythroblastosis vírus* [Salomon *et al.*, 1995; Holbro *et al.*, 2004; Roskoski, 2004], tendo sido o primeiro RTK a ser descoberto [Singh *et al.*, 2005]. Os receptores ErbB são expressos em diversos tecidos de origem epitelial ou a partir de tecidos mesenquimatosos [Singh *et al.*, 2005].

Sob normais condições fisiológicas, a activação dos receptores ErbB é controlada espacial e temporalmente pela família de ligandos EGF [Holbro *et al.*, 2004], estando esta família dividida em 3 grupos. O primeiro grupo inclui EGF, anfirregulina (AR), e factor de crescimento transformador (TGF-α), os quais ligam-se especificamente ao EGFR; o segundo grupo, beta-celulina (BTC), EGF ligado à heparina (HB-EGF), e epirregulina (EPR), ligam-se especificamente a EGFR e ErbB4. O terceiro grupo é composto por neurregulinas (NRG), NRG-1, NRG-2, NRG-3 e NRG-4, que se ligam a ErbB3 e ErbB4 [Holbro *et al.*, 2003].

EGFR está localizado no cromossoma 7p 11-13 [Wells, 1999], sendo uma glicoproteína de membrana composta por um domínio aminoterminal extracelular de ligação do ligando, uma região transmenbranar hidrofóbica e um domínio citoplasmático composto por um domínio tirosina cinase e uma região carboxi-terminal que contém resíduos de tirosina, ocorrendo nesta zona reguladora uma modificação na conformação [Lewin, 2000; Arteaga, 2001].

A estequiometra da ligação para activação do receptor é de 2 mol de EGF para 2 mol de EGFR [Roskoski, 2004]. Quando ocorre dimerização, podem formar-se homodímeros de EGFR ou heterodímeros entre este e os diferentes membros da família [Earp *et al.*, 1995], EGFR interage com ErbB2 ou ErbB3 mas usualmente não dimeriza com ErbB4 [Singh *et al.*, 2005]. A homodimerização ou heterodimerização de ErbB, assim como, a

multiplicidade de ligandos, promove potencialmente uma maior diversidade de sinais [Holbro *et al.*, 2004].

As vias de transdução de sinal são iniciadas, após activação de ErbB, por um conjunto de proteínas como Shc, Grb7, Grb2, Crk, Nck, a fosfolipase Cγ (PLCγ), as cinases intracelulares Src e PI3K, as proteinas tirosina-fosfatases SHP1 e SHP2, e a ubiquitina-ligase Cbl E3 (Fig5). Todos os ligandos e os respectivos receptores de ErbB podem activar a via *ras*/*raf*/MEK/MAPK através das proteínas adaptadoras Grb2 ou Shc. São activados factores de transcrição como c-*fos*, c-*Jun*, c-*myc*; STAT (*signal transducer and activator(s) of transcription*); NF-kB; o factor de transcrição *zinc finger* e membros da família Ets (Fig5) [Normanno *et al.*, 2006].

Actualmente, foi demonstrado que o receptor de EGF pode funcionar como factor de transcrição. Após clivagem da região citoplasmática do receptor, esta move-se para o núcleo podendo afectar a transcrição de genes alvos [Heldin *et al.*, 2001; Waugh *et al.*, 2001; Wells *et al.*, 2002].

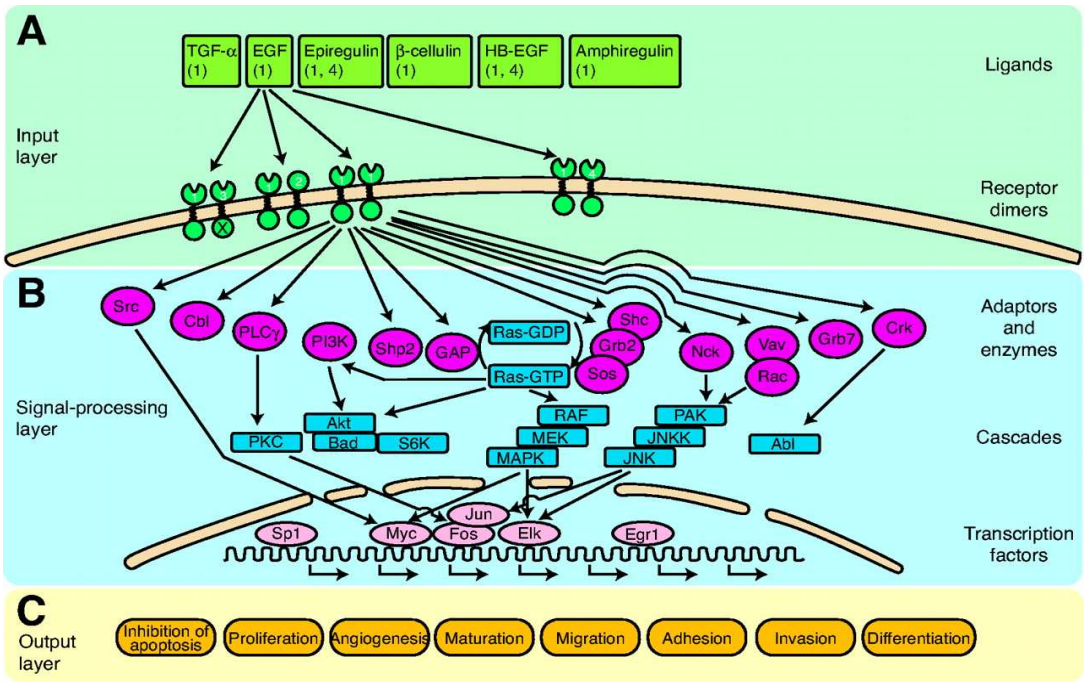

Figura 5. Vias activadas pelo EGFR [Arteaga *et al.*, 2002].

Depois da dimerização dos receptores sucede-se a endocitose, que por possível regulação da sinalização, ocorre degradação do receptor ou reciclagem do receptor voltando este à superfície membranar [Henson *et al.*, 2006].

EGFR pode activar diferentes vias de sinalização, envolvidas na sobrevivência, proliferação, adesão, migração e diferenciação celular [Sedlacek, 2000]. EGF e outros

factores de crescimento estimulam o início da fase G1 da célula. EGFR está envolvido na regulação da ciclina D (promove a passagem da fase G1 para S) [Harari *et al.*, 2000; Quon *et al.*, 2001]. Contudo, ao EGFR podem ligar-se antagonistas, impedindo a ligação do ligando e consequente activação do receptor [Noonberg *et al.*, 2000].

1.1.3.2. Transdução de Sinal e Cancro

Cada componente das cascatas de transdução de sinal está em posição de desencadear modificações nos fenótipos celulares por alterações associadas à multiplicação celular, alterando directamente a expressão genética, constituindo as oncoproteínas [Lewin, 2000].

Proteínas de tirosina-cinase (PTK) quando mutadas ou alteradas estruturalmente, podem tornar-se potentes oncoproteínas, tirosina-cinases oncogénicas (OTKs), estando estas envolvidas na indução de muitos tipos de tumores [Blume-Jensen *et al.*, 2001; Skorski, 2002]. OTKs têm dois papéis complementares no cancro: estimulam as vias de sinalização que promovem a independência celular relativamente ao ambiente; tornam as células mais resistentes à quimioterapia e radioterapia [Skorski, 2002].

A promoção de tirosina-cinases oncogénicas ocorre por quatro principais mecanismos genéticos: i) rearranjos genómicos, como translocações cromossomais, que podem ter como resultado a fusão de proteínas, originando PTK activas contitutivamente; ii) sobre-expressão, resultante da amplificação de genes, levando à espontânea dimerização; iii) mutações pontuais ou pequenas deleções, causando constitutiva independência do ligando; iv) truncamento do terminal carboxil, sucedendo uma conformação activa da PTK [Blume-Jensen *et al.*, 2001; Skorski, 2002]. Em geral, o efeito da tranformação dos oncogenes pode facultar o aumento ou a activação constitutiva da actividade cinásica, promovendo uma alteração na sinalização quer quantitativamente quer qualitativamente [Blume-Jensen *et al.*, 2001]. Estas alterações podem conduzir à transformação para cancro, dado que as OTKs podem induzir proliferação incontrolada, inibição da apoptose, inibição da diferenciação e/ou desregulação da adesão [Skorski, 2002]. OTKs podem induzir resistência às drogas citostáticas e à irradiação, tornando as curas raras nos tumores positivos a OTK, o que se propõe como estratégia adicionar inibidores de OKT às terapias tradicionais [Skorski, 2002].

A sobre-expressão dos factores de crescimento pode ter como procedência: *via autócrina*, em que a própria célula secreta factores de crescimento, promovendo o seu

crescimento através de um *feedback* positivo; *via parácrina,* em que as células do estroma secretam os factores de crescimento promovendo a proliferação das células adjacentes [Ciardiello et al., 2001; Arteaga et al., 2002].

A título de exemplo, a sobre-expressão é evidente na forma EGFR em 80% dos carcinomas de células escamosas do pulmão, 50% em antrocitomas, 80-100% em tumores da cabeça e pescoço e com menor frequência nos carcinomas da bexiga e gastrointestinais. As formas ErbB2 estão amplificadas em 25% dos tumores da mama e nos adenocarcinomas do ovário, pulmão, estômago e glândulas salivares. A mais comum das proteínas transdutoras de sinal pertence à família Ras e está presente em 15 a 20% de todos os tumores humanos sob diversas formas mutadas (excepcionalmente, nos tumores cervicais e da mama, onde estas mutações são raras). As tirosina-cinases não receptoras, raramente estão activadas nos tumores humanos, com excepção da c-ABL (*Abelson Murine Leukemia Viral Oncogene*). Também, algumas células neoplásicas adquirirem a capacidade de sintetizar factores de crescimento, para actuar em si próprias. O oncogene *Ras* constitui um exemplo, estimulando a expressão de grandes quantidades de factores de crescimento tais como TGF-α. O factor de transcrição mais comum nos tumores é a oncoproteína Myc [Kumar *et al.* 2003].

1.1.4 Factor de Crescimento Epidérmico – EGF

O EGF foi inicialmente isolado por Cohen em glândulas submaxilares de rato [Cohen, 1962]. É um péptido com 53 a.a., sintetizado a partir de uma proteína percursora de 1168 aminoácidos [Gray *et al.*, 1983] e exibe 3 pontes de enxofre (S-S) [Goustin *et al.*, 1982]. O EGF é igua1mente conhecido por Urogastrom; este último foi descoberto depois de EGF mas só mais tarde se constatou serem a mesma molécula [Smith *et al.*, 1982, Fisher *et al.*, 1990]. O seu gene localiza-se no braço longo do cromossoma 4 entre o locus 25 e 27 (4q 25→27) [Morton *et al.*, 1986]. É um factor mitogénico em diversos tipos de tecidos de origem mesenquimatosa ou epitelial [Goustin *et al.*, 1983].

O EGF pertence à família de factores de crescimento de EGF, sendo o EGF e TGF-α os principais péptidos que se ligam ao EGFR [Fisher *et al.*, 1990]. Apesar de terem uma

acção similar, o TGF-α é mais específico como estimulador da angiogénese e na libertação de cálcio do osso [Fisher *et al.*, 1990]. Ao EGF são-lhe atribuídas múltiplas funções tais como: estimulação da proliferação de células epiteliais [Carpenter *et al.*, 1979], uma vez que constitui um importante factor mitogénico; diferenciação de células específicas, podendo esta estar associada à activação de cAMP [Knecht *et al.*, 1983]; inibição da secreção gástrica [Urdea *et al.*, 1983]; promoção da diferenciação do céu da boca e pele; crescimento dos olhos, erupção dos dentes e folículos do cabelo; maturação dos pulmões; crescimento do intestino e fígado; diferenciação dos neurónios [Fisher *et al.*, 1990]; estimulação da polimerização da actina [Boonstra *et al.*, 1995]. O EGF está igualmente envolvido na manutenção do estômago, intestinos e na integridade do epitélio urinário, facto relacionado com a sua presença na saliva e urina [Fisher *et al.*, 1990]. Estas funções são mediadas através das vias autócrina, parácrina e endócrina [Boonstra *et al.*, 1995].

Os níveis de EGF variam em diferentes fases da vida. Foi observado um aumento significativo da sua concentração na glândula submandibular e no plasma de fêmeas de rato durante a gestação [Tsutsumi *et al.*, 1987]. Contudo, os níveis de EGF ou do seu precursor estão em baixos níveis nos tecidos fetais de ratos e após a puberdade, embora sejam elevados durante o período neo-natal [Fisher *et al.*, 1990]. Nos seres humanos o EGF é detectado em diversos órgãos a partir das 20 semanas de gestação [Mattila *et al.*, 1987].

Em diferentes tecidos a concentração de EGF depende da acção hormonal. O aumento da sua concentração nos tecidos, sangue e urina está associado ao aumento das hormonas da tiróide e sexuais, causando estas últimas maior estabilidade ao mRNA precursor de EGF. Por sua vez o EGF estimula a secreção de diversas hormonas, nomeadamente hipotalâmicas e pituitárias hipofisárias, e inibe secreções hormonais da tiróide, testículos e ovários [Fisher *et al.*, 1990]. Nos seres humanos, a tiróide aumenta os níveis de EGF na urina mas não exerce efeitos na saliva ou no corpo [Mattila, 1987] o que permitiu concluir que o EGF tem efeito autócrino e/ou parácrino [Fisher *et al.*, 1990].

1.1.4.1 Polimorfismos no Gene *EGF*

Variações na sequência de um gene presentes em mais de 1% da população são designados polimorfismos [Brookes, 1999] e, se esta variação ocorrer num único nucleótido é designada por SNP (*single nucleotide polymorphism*) [Erichesen *et al.*, 2004].

Shahbazi *et al.* (2002) identificaram um polimorfismo no nucleótido 61 do gene *EGF* que consiste na substituição da base adenina (A) por uma guanina (G). A posição 62 está associada à ligação do factor nuclear (NF)-kB, podendo afectar o enrolamento do DNA ou o processamento da transcrição do mRNA. Os mesmos autores confirmaram que o polimorfismo era funcional. Em homozigóticos para A ocorre menor produção de EGF que nos homozigóticos para G ou heterozigóticos [Shahbazi *et al.*, 2002]. Segundo Shahbazi, os melanomas malignos estão associados a um aumento de risco comparando o genótipo G/G com o genótipo A/ A [Shahbazi *et al.*, 2002].

1.1.5 Cancro da Mama

É o cancro mais comum na mulher; com 1,15 milhões de casos em 2002. Mais de metade dos casos são registados em países industrializados, destacando-se a América do Norte e a Europa. O Japão constitui uma excepção a esta regra [Parkin *et al.*, 2005] (Fig6).

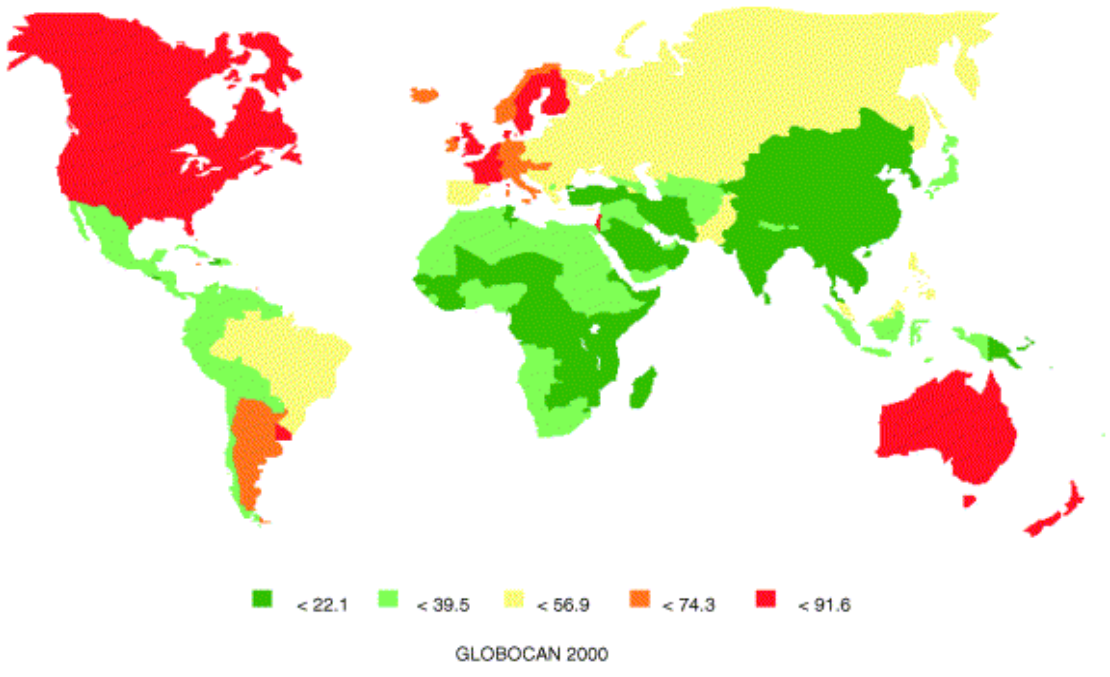

Figura 6. Incidência de cancro na mama (por 100 000 habitantes). (Adaptado de [Parkin *et al.*, 2001]

Este modelo oncológico incorpora os seguintes factores de risco [Kumar *et al.*, 2003]: *idade*- o risco aumenta com a idade; *idade de menarca e menopousa*- o risco aumenta com

menarca antes dos 11 anos e com menopausa tardia; *primeiro nado vivo*- mulheres com a primeira gestação antes dos 20 anos, têm metade do risco relativamente a mulheres que têm a primeira gestação acima dos 35, ou que são nulíparas; *parentesco de primeiro grau com indivíduos que apresentaram cancro da mama*- 13% das mulheres com este carcinoma tiveram um parente de primeiro grau afectado; *raça*- as mulheres caucasianas têm maior incidência que as afro-americanas, apresentando este último grupo, por sua vez, um estagio mais avançado e um índice de mortalidade superior (as mulheres negras não apresentam receptores hormonais e possuem diferentes alterações no gene *p53*)**;** *exposição ao estrogénio*- a reposição hormonal pós-menopausa aumenta ligeiramente o risco de desenvolvimento de carcinoma; *exposição à radiação*- o risco é superior após exposição radiação terapêutica ou à radioactividade nuclear; *dieta*- uma dieta rica em gordura poderá aumentar o risco, em oposição, a ingestão de β-carotenos poderá diminui-lo, assim como o consumo moderado ou acentuado de álcool está associado a um elevado risco; *obesidade*- o risco é baixo em mulheres obesas menores que 40 anos e alto para mulheres obesas pós-menopausa; e a *amamentação no peito*- quanto maior o período de amamentação menor o risco [Kumar *et al.*, 2003].

Dentro dos casos com risco familiar, aproximadamente 3% apresentam mutações nos genes autossómicos dominantes altamente penetrantes, *BRCAl* e *BRCA2*. Mulheres com estas mutações têm um risco de 60-85% de vir a desenvolver cancro da mama [Kumar *et al.*, 2003].

As alterações moleculares mais frequentes são dos genes supressores, que ocorrem ao nível de mutações em *p53* e *Rb*, e metilação no *p16*. Os proto-oncogenes mais afectados, promovidos por amplificação, estão ao nível do receptor ErbB2, no factor de transcrição Myc e no regulador do ciclo celular (ciclina Dl) [DeVita *et al.*, 2001].

Os carcinomas da mama são classificados em carcinomas *in situ* e invasivos. O primeiro grupo subdivide-se em carcinoma ductal *in situ* e carcinoma lobular *in situ*, dependendo se a proliferação das células tumorais se dá no interior dos ductos ou dos lóbulos. Os carcinomas invasivos integram o carcinoma ductal invasivo (79% de todos os casos), carcinoma lobular invasivo, carcinoma medular, carcinoma mucinoso, carcinoma tubular e carcinoma papilar [Robbins *et al.*, 1999].

1.6 Cancro do Ovário

O cancro do ovário é o sexto cancro mais comum e a sétima causa de morte por cancro. No ano 2002 ocorreram 204 000 novos casos e 125 000 mortes. Os valores de incidência são mais altos nos países desenvolvidos, excepto no Japão [Parkin *et al.*, 2005].

A classificação destes tumores baseia-se no tecido de origem mais prováve1, podendo considerar-se três tipos: tumores epiteliais, de células germinativas e dos cordões sexuais/estroma [Kumar *et al.*, 2003].

Os factores de risco envolvidos neste tipo de cancro não se encontram tão bem estabelecidos como para os outros tumores dos órgãos reprodutores. A nuliparidade é um factor de risco. Mulheres solteiras têm maior incidência que mulheres casadas com paridade reduzida, assim como mulheres com história de disgenesia gonadal. Têm menor risco as mulheres que, entre os 40 e 59 anos, tomaram contraceptivos orais. Para mulheres portadoras das mutações *BRCAl* e *BRCA2* o risco é de 20 a 60% [Kumar *et al.*, 2003].

A nível molecular 50% destes carcinomas tem o gene *p53* mutado e 30% dos adenocarcinomas expressam elevados níveis do oncogene ErbB2 [Kumar *et al.*, 2003].

1.7 Cancro do Colo do Útero

O cancro no colo do útero é a segunda neoplasia maligna mais frequente no sexo feminino. Estima-se que ocorreram 493 000 novos casos e 274 000 mortes no ano 2002. É muito mais comum nos países em desenvolvimento. As taxas de mortalidade são muito baixas e estão a decrescer substancialmente nos países orientais devido à prática de programas de rastreio [Parkin *et al.*, 2005] (Fig7).

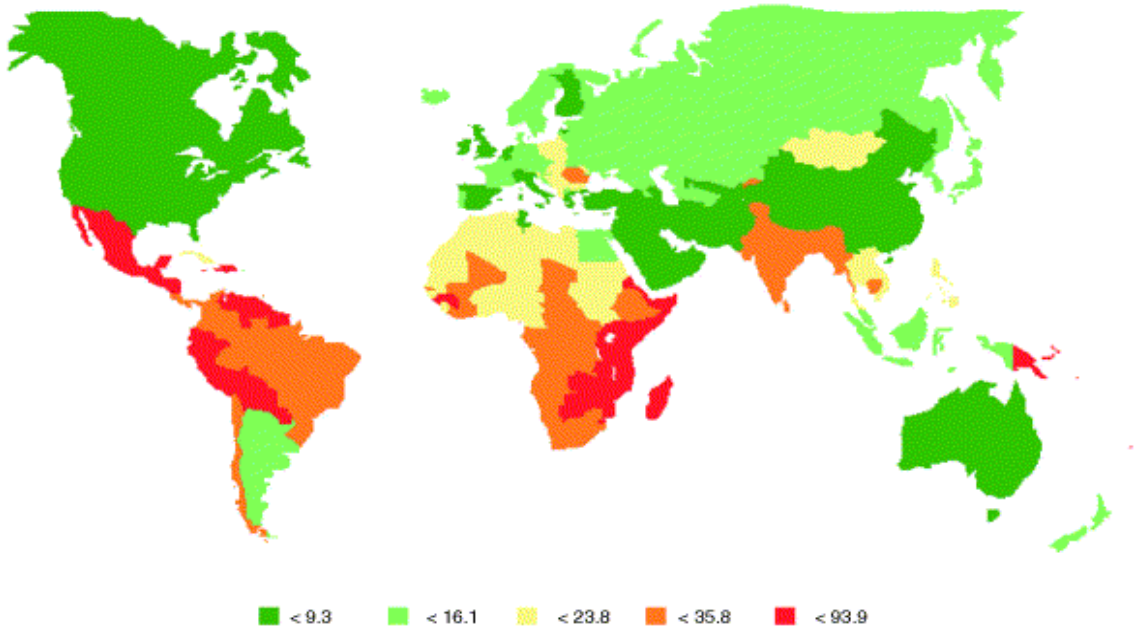

Figura 7. Incidência de cancro do colo do útero (por 100 000 habitantes). (Adaptado de [Parkin *et al.*, 2001]

Estudos epidemiológicos apontam como importante factor causal do carcinoma cervical um vírus transmitido sexualmente, o HPV [DeVita *et al.*, 2001; Kumar *et al.*, 2003]. Existem mais de 100 subtipos de HPV, tendo sido identificados os subtipos 16, 18, 31, 33 e 45 como de alto risco [DeVita *et al.*, 2001; Kumar *et al.*, 2003]. Pensa-se que a infecção por HPV seja necessária mas insuficiente para causar o cancro. As lesões CIN (neoplasia intra-epitelial cervical) de alto grau e o carcinoma invasivo requerem infecção persistente. Vários factores podem facilitar a infecção persistente, integração do DNA e progressão da doença, tais como o uso de contraceptivos orais, paridade elevada, e o fumo de tabaco [DeVita *et al.*, 2001]. De um modo geral, os factores de risco para a neoplasia cervical apontam para início muito precoce da actividade sexual, vários parceiros sexuais, parceiro sexual que teve relações sexuais com diferentes parceiros, grande número de gestações, exposição a HPV de alto risco, exposição a contraceptivos orais e nicotina, e co-infecções genitais [Kumar *et al.*, 2003].

A nível molecular, o HPV produz as proteínas E6 (proteína ubiquitina ligase E3A) e E7 (factor associado ao retinoblastoma 600), que interrompem o ciclo celular das células do hospedeiro (E7), interrompem a apoptose (E6) ou induzem a duplicação dos centrossomas (E6, E7). A proteína E6 induz a degradação da proteina p53 por protólise pela via da ubiquitina, reduzindo a sua quantidade até 2 a 3 vezes. A proteína E7 liga-se à

forma hipofosforilada da proteína Rb (agente inibidor da entrada na fase S, devido à sua ligação ao factor de transcrição E2F), conduzindo a sua protólise. A Rb hipofosforilada ligar-se-ia ao factor de transcrição E2F, inibindo deste modo a entrada na fase S [Kumar *et al.*, 2003]. A proteína E6 com alta afinidade para p53 está associada aos tipos oncogénicos de alto risco [DeVita *et al.*, 2001]. Estas proteínas alteram os mecanismos normais do controlo do ciclo celular, aumentando o tempo de vida das células, situação que promove o desenvolvimento de tumores [DeVita *et al.*, 2001; Kumar *et al.*, 2003].

A maioria dos carcinomas do colo do útero é precedida por uma lesão precursora, sendo o rastreio fundamental no diagnóstico precoce do cancro cervical. A lesão pré-cancerosa, classificada com base no grau de maturação epitelial e a distribuição da atipia citológica [Robbins *et al.*, 1999; DeVita *et al.*, 2001], pode manter-se durante muito tempo, ou regredir. A evolução para carcinoma aumenta conforme a gravidade das alterações pré-cancerosas; estas, por sua vez, estão associadas aos tipos de HPV de alto risco [Kumar *et al.*, 2003]. Contudo, as taxas de progressão não são uniformes [Robbins *et al.*, 1999]. Nas lesões CIN de baixo grau a atipia encontra-se nas camadas superficiais com preservação da maturação epitelial enquanto nas lesões CIN de alto grau a atipia afecta a camada superficial e a camada de células basais, com diminuição da maturação. No carcinoma *in situ* a atipia é evidente em todas as camadas, com ou sem maturação mínima [Robbins *et al.*, 1999; Kumar *et al.*, 2003].

O carcinoma invasivo tem como precursor o carcinoma *in situ* e, esta progressão ocorre em 70% das mulheres sem tratamento. Sob o ponto de vista histológico os tumores são constituídos por células grandes moderadamente bem diferenciadas e não queratinizadas (65%), células grandes queratinizadas (25%) e pequenas células escamosas indiferenciadas (10%) [Robbins *et al.*, 1999].

1.2 OBJECTIVOS

Este estudo pretendeu avaliar a existência de associação entre o polimorfismo funcional A61G do gene *EGF* e a predisposição para o desenvolvimento de tumores sólidos, nomeadamente da mama, ovário e colo do útero.

Esta dissertação está organizada em 6 partes. No primeiro capítulo foi feita uma introdução geral ao tema e aos objectivos do trabalho. O segundo e terceiro capítulo contêm, de forma simplificada, os métodos e os resultados apresentados nos documentos em anexo. O quarto capítulo apresenta a discussão e perspectivas futuras referentes a todo o trabalho realizado e expresso nos três artigos apresentados em anexo. O quinto capítulo contém as referências bibliográficas utilizadas na execução desta dissertação. O capítulo final contém três anexos referentes a três artigos submetidos e elaborados com a informação produzida neste estudo.

2. MÉTODOS

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2.1 Obtenção das Amostras

No presente trabalho realizou-se um estudo do tipo caso-controlo em 1442 mulheres. Num total de 942 indivíduos com neoplasia, 175 casos compreendiam cancro do ovário, 383 de cancro da mama e 384 manifestavam lesões do colo do útero. O grupo controlo constituído por 500 indivíduos do sexo feminino sem doença oncológica, apresentaram uma mediana de 41 anos.

O grupo de mulheres com cancro do ovário, apresentaram a mediana de 53 anos e expunham em 57% dos casos o tipo histológico seroso; 15% células claras; 14% mucinoso; 10% endometrioide e 4% outros tipos histológicos. Os casos de cancro da mama, com mediana de 44 anos, apresentaram 89,0% carcinoma ductal invasor; 4,2% carcinoma lobular invasor e 6,8% outros tipos histológicos. Nos indivíduos com lesões cervicais a mediana é de 35 anos, nos quais 233 indivíduos possuíam cancro cervical (mediana 47 anos), com 78,4% o tipo histológico espinocelular, 12,4% adenocarcinoma e 3,9% outros tipos histológicos.

2.2 Análise do Polimorfismo A61G do Gene *EGF*

Foram recolhidos cerca de 8ml de sangue periférico dos indivíduos supra referidos, através de colheita intravenosa, para tubos contendo EDTA ($Na_2H_2Y.2H_2O$). O isolamento de DNA procedeu-se a partir de células nucleadas do sangue periférico, recorrendo-se, para tal, à técnica mista *salting-out-cloroformio* [Mullenbach *et al.*, 1989].

A região do gene *EGF* que contem o polimorfismo foi amplificada através da técnica PCR. O fragmento amplificado possui 242 pares de bases (pb) [Shahbazi *et al.*, 2002] e a reacção foi processada num termociclador [Biometra®]. A mistura reaccional foi constituída por cerca de 0,2 μg de DNA genómico, 5 μl de tampão de reacção [Mbi Fermentas], 1 U de Taq DNA polimerase [Mbi], 1,5 mM de $MgCl_2$ [Mbi], 0,2 mM de dNTPs [Mbi] e 0,3 μM de primeres específicos para a região do gene pretendida (Forward: 5'-TGT CAC TAA AGG AAA GGA GGT-3' e Reverse: 5'-TTC ACA GAG TTT AAC AGC CC-3'), perfazendo um

volume final de 50 µl. As condições da reacção incluíram um passo de pré-desnaturação a 95°C durante 5 minutos, seguido de 38 ciclos (cada um dos ciclos com três passos de 94°C durante 1 minuto, 55°C durante 1 minuto e de 72°C durante 1 minuto) e um passo de extensão final de 72°C durante 5 minutos.

A identificação do fragmento de DNA foi realizada pela técnica de electroforese, em géis de agarose a 3% (p/v), que foram corados com brometo de etídeo e visualizados sob luz ultravioleta, através do equipamento Image Master VDS [Pharmacia Biotech]. Utilizou-se 13µ1 da amostra amplificada; o gel foi corado com 10 mg de brometo de etídeo por ml de gel; e foi usada a solução TBE ($H_2NC(CH_2OH)_3$ 10,9% (p/v), H_3BO_3 5,55% (p/v), $C_{10}H_{14}N_2O_8Na_2.2H_2O$ 0,93% (p/v) [Merck]) na preparação dos géis e como tampão de electroforese. Para verificar a amplificação com o tamanho pretendido, comparou-se o produto com um marcador molecular de peso (100 pb) [Mbi].

O polimorfismo A61G do gene EGF foi analisado através da técnica RFLP. Da amostra amplificada, 10µl foram submetidos a digestão enzimática com 2U da enzima de restrição *Alu*I [Fermentas] e com o respectivo tampão de reacção finalizando um volume de 15µ1. A digestão realizou-se durante a noite a 37°C.

Os fragmentos obtidos por RFLP foram submetidos a electroforese em géis de agarose 3% (p/v), utilizando o mesmo procedimento para a análise dos produtos de PCR. A enzima corta o produto de PCR no nucleótido 61, se o alelo A estiver presente. Deste modo são esperados os seguintes fragmentos: para o alelo A 15, 34, 91 e 102 pb e para o alelo G 15, 34, 193 pb.

2.3 Análise Estatística

Todos os resultados obtidos foram tratados estatisticamente recorrendo aos programas informáticos SPSS 13® (Statistical Package for Social Sciences) e EPI INFO6®.

A comparação das frequências obtidas para os diferentes grupos em estudo foi realizada com recurso ao teste do Qui-quadrado (χ^2). O valor de p obtido pelo teste do χ^2 é considerado estatisticamente significativo quando inferior a 0,05.

O valor de *Odds Ratio* (OR) e o seu Intervalo de Confiança para 95% (95% CI), permitem inferir se o polimorfismo em estudo é um factor de risco ou um factor protector para o desenvolvimento de tumores, comparativamente a indivíduos controlo. Assim, se o valor de OR e o respectivo CI for superior a 1 há uma associação positiva com o desenvolvimento de cancro, se for inferior 1 estamos perante uma associação negativa para o desenvolvimento de cancro.

Para a comparação de variáveis contínuas, como médias e desvios-padrão, recorreu-se ao teste de *t-Student*.

Foi também analisado o tempo de espera para aparecimento de doença (*waiting time to onset of disease* (WTO)), sendo definido como o intervalo entre o tempo de exposição do factor de risco (A61G) e o tempo de aparecimento de doença, utilizando a metodologia de *Kaplan-Meier*.

3. RESULTADOS

3. RESULTADOS

Neste capítulo apenas estão apresentados, de modo sucinto, os resultados estatisticamente significativos, dos artigos apresentados em anexo (pág. 70, 90 e 109).

3.1 Cancro do Ovário

No grupo de indivíduos com cancro do ovário, os portadores do alelo G apresentam menor risco de vir a desenvolver esta neoplasia (OR=0,72; 95%CI: 0,55-0,94; p=0,012), sendo corroborado pela análise de regressão logística ajustado à idade. Observou-se também uma tendência linear na avaliação de risco para a presença de dois, um ou nenhum alelo G (p = 0,014). Quando subdividida a amostra o grupo de mulheres com idade inferior a 53 anos apresenta igualmente o mesmo efeito protector (OR=0,63; 95%CI: 0,43-0,91; p=0,009), assim como o grupo de pessoas diagnosticadas com doença avançada (estadio III e IV) (OR OR=0,63; 95%CI: 0,45-0,89; p=0,006). Observou-se também que nos indivíduos portadores do genótipo GG a idade média para aparecimento da doença é mais tardia que os portadores do genótipo AA (p = 0,035).

3.2 Cancro da Mama

Mulheres com cancro da mama e portadoras do alelo G apresentam menor risco de desenvolver esta neoplasia (OR=0,82; 95% CI: 0,08-1,00; p=0,012), apresentando este subgrupo mais tarde a média de aparecimento de doença relativamente às portadoras do alelo A (p = 0,041).

3.3 Cancro do Colo do Útero

Nas mulheres com cancro cervical, portadoras do genótipo G, com idades inferiores a 47 anos e com doença avançada apresentam maior risco de desenvolver esta neoplasia relativamente ao restante grupo (OR=3,17; 95%CI: 1,21-8,26; p=0,016).

4. Discussão e Considerações Finais

4. Discussão e Considerações Finais

A predisposição para cancro pode ocorrer através da combinação de variações genéticas de fraca penetrância, podendo estas ter maior significado para saúde pública, relativamente aos marcadores familiares de risco [Balmain *et al.*, 2003]. A maioria destas variações genéticas são polimorfismos de um único nucleótido (SNP) [Ponder, 2001], e podem consistir em variações de genes envolvidos na metabolização de carcinógenos, na reparação do DNA, na expressão de proto-oncogenes e supressores tumorais [Harris, 1991; Bartsch *et al.*, 1996]. Embora os carcinomas estejam fortemente associados às exposições ambientais, os polimorfismos genéticos podem modificar o efeito dessas exposições, contribuindo ambos no peso para a ocorrência de cancro. Contudo, uma avaliação da penetrância dos polimorfismos genéticos poderá implicar novos alvos clínicos e novas estratégias de saúde pública, relativamente à prevenção e controle do cancro [Rothman *et al.*, 2001].

O presente estudo apresenta a associação entre o polimorfismo funcional A61G do gene *EGF* e o desenvolvimento para cancro em três tumores sólidos, numa amostra populacional do Norte de Portugal. Foram estudados três modelos oncológicos, incidentes na mulher, com diferentes etiopatogenias: hormonal, no cancro da mama e ovário; e viral, no cancro do colo do útero.

O estudo é pioneiro nos resultados apresentados, sendo que nunca estudado no cancro do ovário. Este estudo foi iniciado por Shabhazi *et al.* (2002) em melanoma maligno, no qual foi identificado o polimorfismo A61G como sendo funcional. Este estudo, demonstrou que em culturas de células mononucleadas do sangue periférico, os indivíduos portadores do alelo G têm maior produção de EGF, relativamente aos portadores AA. Esta alteração transcricional poderá resultar de um factor nuclear (NF)-κB, com possível ligação à posição 62 [Shabhazi *et al.*, 2002]. Estudos posteriores têm vindo a analisar a associação do referido polimorfismo, sendo em número crescente os modelos estudados. De referir os primeiros estudos realizados em melanomas e cancro gástrico, ocorrendo em ambos uma divergência de resultados, quanto à susceptibilidade aumentada para cancro em portadores G [Shahbazi et al., 2002; Mccarron et al., 2003; Amend et al., 2004; Hamai et al., 2004; James et al., 2004; Goto et al., 2005].

4.1 EGF e Cancro

Os factores de crescimento activam os complexos processos de sinalização, promovendo alterações celulares [Mosesson *et al.*, 2004; Bublil *et al.*, 2007]. Correspondem a *sinais positivos* na estimulação celular, sendo regulados por *sinais negativos*, que controlam a sua amplitude e duração. O balanço entre ambos os sinais é crucial para a homeostasia da célula [Mosesson *et al.*, 2004]. O factor de crescimento epidérmico (EGF) desempenha um papel chave na promoção da sobrevivência celular [Henson *et al.*, 2006]. Após a ligação ao seu receptor (EGFR), activa um conjunto de cascatas transdutoras de sinal, culminando na alteração da expressão genética [Clague *et al.*, 2001; Holbro *et al.*, 2004; Henson *et al.*, 2006]. A activação do EGFR não é importante apenas na proliferação celular, contribuindo igualmente noutros processos envolvidos na progressão para cancro, como angiogénese, metastização e inibição da apoptose [Ciardiello *et al.*, 2001].

Actualmente, sabe-se que os receptores do grupo ErbB estão envolvidos no desenvolvimento de diversos tipos de cancro [Hynes *et al.*, 2005]. O aumento dos sinais activados pelo EGFR pode dever-se ao aumento da expressão do EGFR, aumento da concentração dos ligandos (via autócrina ou parácrina), presença de receptores alterados por mutação genética, e modificações nas moléculas reguladoras destes receptores [Ciardiello *et al.*, 2001; Arteaga *et al.*, 2002].

Alguns estudos no cancro da mama, ovário e colo do útero têm sugerido que os tumores que apresentam alterações no EGFR estão associados a doença mais agressiva, apresentando pior prognóstico clínico [Arteaga *et al.*, 2002; Holbro *et al.*, 2003; Hynes *et al.*, 2005].

4.1.1 Polimorfismo A61G no Gene *EGF* e Cancro do Ovário e Mama

Quando analisados os resultados, observou-se que os portadores do alelo G estão associados a um efeito protector no cancro do ovário e da mama. No caso do cancro do ovário os portadores do alelo G têm menor risco de desenvolver esta neoplasia (OR=0,72;

p=0.012), estando este alelo menos presente nas mulheres mais novas (≤53 anos) (OR=0,63; p=0.009), assim como nas pacientes diagnosticadas com doença avançada (OR=0,63; p=0.006). Observou-se também, que em portadores do genótipo GG, a doença surge mais tarde em comparação com portadores AA, segundo a análise de *Kaplan-Meier* (p=0,035). Relativamente à análise realizada no carcinoma da mama, os portadores do alelo G têm menor risco de desenvolver esta doença (OR=0,82; p=0,043), apresentando também, esta doença mais tarde segundo a análise de *Kaplan-Meier* (p=0,041).

O EGFR forma heterodimeros com outros receptores ErbB [Holbro *et al.*, 2003], permitindo assim, um aumento na diversidade de ligandos reconhecidos, e consequentemente um aumento do reportório das vias de sinalização que podem ser activadas [Roskoski 2004]. Sabe-se que EGFR interage directamente com a ubiquitina ocorrendo degradação lisossomal, após internalização do receptor e do ligando, contudo, o receptor pode voltar à superfície da célula. A co-expressão dos receptores ErbB interligada com os ligandos exógenos, pode permitir modular a magnitude da resposta [Arteaga 2001]. Assim, a persistência das interacções ligando-receptor controla o tráfego do receptor [Resat *et al.*, 2003]. Este tráfego apresenta as seguintes funções: i) controlar a magnitude do sinal; ii) controlar a especificidade da resposta; iii) controlar a duração da resposta [Resat *et al.*, 2003]. Adicionalmente, sabe-se que o EGFR é um sinalizador com efeito pleiotrópico. Após activação do EGFR as respostas variam de mitogénese a apoptose, migração a differenciação, entre outras [Wells *et al.*, 1999]. Estes diferentes efeitos estão associados à duração e força dos sinais, sendo regulados por mecanismos negativos de regulação, regulação esta, das diferentes vias de sinalização, promovida pela: i) disponibilidade do ligando para o receptor; ii) regulação negativa por fosfatases que podem interferir na amplitude e cinética dos sinais dos receptores; iii) inactivação de sinais através da internalização e degradação do receptor. Este controle é movido pelos diferentes arranjos ligando-receptor, as diferentes combinações de dimerização do receptor, assim como estado e contexto da célula [Singh *et al.*, 2005].

O efeito protector que se observou nos portadores do alelo G, pode ser explicado pela internalização e consequente degradação do EGFR, após a activação do EGF [Wells *et al.*, 1999; Singh *et al.*, 2005]. De acordo com esta hipótese, o complexo EGF/EGFR é internalizado, através de um mecanismo endossomal. Contudo no endossoma o EGF não é

sensível ao pH ácido, e não se dissocia do receptor, não permitindo a reciclagem do mesmo para a superfície celular [Wells *et al.*, 1999; Singh *et al.*, 2005]. É interessante observar que o EGFR é reciclado quando tem como ligando o TGF-α, ou se estiver ligado ao EGF mas quando associado ao ErbB-2 ou ErbB-3 [Lenferik *et al.*, 1998]. Na corroboração desta teoria, outro estudo demonstra um abrupto decréscimo na expressão de EGFR na superfície, quando é adicionado determinadas concentrações de EGF, a linhas celulares [Salazar *et al.*, 2002; Friedman *et al.*, 2005]. Outro estudo também demonstrou que altas concentrações de EGF induzem a perda de adesão, bloqueio do ciclo celular, apoptose e inibição da proliferação em linhas celulares [Zhao *et al.*, 2006]

O desenvolvimento carcinogénico ocorre através de progressivos danos genéticos e epigenéticos, e consequentemente alterações na susceptibilidade para ocorrer proliferação celular, através da activação de proto-oncogenes e/ou inactivação de genes supressores tumorais [Harris, 1991]. A promoção tumoral resulta da proliferação e/ou sobrevivência de células iniciadas, nas quais existe um aumento da probabilidade para ocorrência de alterações cromossómicas, amplificação de genes e alterações na expressão de genes [Harris, 1991]. A sobre-expressão do EGFR conjunta com a activação das suas vias, é crucial na progressão para cancro [Ciardiello *et al.*, 2001; Johnson *et al.*, 2004]. Admitindo a hipótese que ocorre internalização e degradação do EGF e EGFR, portadores do alelo G poderão ter menor expressão de EGFR à superfície da célula, durante a promoção tumoral, porque eles produzem mais proteína EGF, e consequentemente mais EGFR é internalizado e degradado. Como resultado, os portadores G poderão ter menos expressão de EGFR durante a promoção tumoral, o que poderá explicar a protecção para desenvolver cancro.

O EGF exerce acção sobre o ovário [Ozcakir *et al.*, 2005], e dado que ao longo da vida do indivíduo o ovário está exposto ao EGF, pode-se explicar o facto do alelo G estar associado ao aparecimento de doença mais tarde. Portanto, portadores do alelo G podem estar expostos a uma menor expressão de EGFR na superfície da célula, e consequentemente menor activação da cascata de transdução de sinal.

Contudo, também se observou um efeito protector nos indivíduos que são diagnosticados com doença mais avançada. De acordo com Lassus *et al.*, (2006), os estadios III e IV apresentam maior sobre-expressão de EGFR, relativamente aos estadios I

e II. Dado os estadios mais altos apresentarem sobre-expressão de EGFR, novamente, e de acordo com o exposto acima, pode-se explicar os resultados no facto dos portadores do alelo G poderem expressar menor EGFR.

Este efeito protector também foi observado nas mulheres mais novas (<53 anos). O EGF é expresso no ovário [Leung *et al.*, 1992; Ozcakir *et al.*, 2005], e actua na evolução dos folículos dos ovários [Marui *et al.*, 1993; Ashkenazi *et al.*, 2005; Conti *et al.*, 2006]. Contudo o EGF inibe a concentração das hormonas produzidas nos ovários (estrogéneo e progesterona) e decresce a expressão de FSH (*folicule-stimutating*) [Hsueh *et al.*, 1981; Jones *et al.*, 1982; Pulley *et al.*, 1986; Findlay *et al.*, 1999]. Uma vez que a mulher mais nova está exposta às hormonas sexuais, e estas estão, por sua vez, associadas a uma maior proliferação celular, um decréscimo destas hormonas promovido pelos portadores do alelo G, reduz o risco para o desenvolvimento desta neoplasia.

No cancro da mama também foi observado um efeito protector, em portadores do alelo G. O EGF quando comparado com outros ligandos, apresenta distintos eventos biológicos, e as suas acções podem ser condicionadas pela presença de diferentes vias intracelulares, promovidas pela *down-regulation* do EGFR [Taketani *et al.*, 1983]. De facto, é encontrada plausibilidade biológica, no efeito protector dos indivíduos portadores do alelo G, sugerida pelos mecanismos de activação do EGF, e consequentemente internalização e degradação do seu receptor [Friedman *et al.*, 2005], devido a uma possível maior produção de EGF nos portadores do alelo G.

A evidência da literatura sugere que o EGF está envolvido no desenvolvimento da mama [Taketani *et al.*, 1983; Fisher *et al.*, 1990; Spitzer *et al.*, 1995; Rosfjord *et al.*, 1999; Dehnhard *et al.*, 2000], nomeadamente na estimulação do epitélio ductal e alveolar [Fisher *et al.*, 1990; Spitzer *et al.*, 1995]. O EGF também foi localizado no interior dos lóbulos e glândulas da mama [Snedeker *et al.*, 1991], estando presente no leite, pois é secretado no tecido da glândula mamária durante a lactação [Fisher *et al.*, 1990], sendo mesmo encontrado em altas concentrações depois do desmame [Beardmore *et al.*,1983; Moran *et al.*, 1983; Brown *et al.*, 1989; Fisher *et al.*, 1990]. Deste modo o EGF está associado ao tecido mamário ao longo da vida, sendo observada uma protecção para risco. É

interessante observar que o cancro da mama está associado a um decréscimo de risco com a amamentação, inclusive com idades abaixo dos 24 anos [Russo *et al.*, 2005].

Adicionalmente, os heterodimeros EGFR/ErbB2 estão associados a sinais mais robustos e mitogenicamente mais activos relativamente aos homodimeros EGFR [Arteaga, 2001; Roskoski, 2004], e na ocorrência de alterações nestes receptores os tumores estão associados a maior agressividade [Holbro *et al.*, 2003]. Assim, pode-se observar que outros factores estão fortemente associados ao cancro da mama, além do EGF, podendo executar influência, nomeadamente, para progressão de doença. Por outro lado, foi demonstrado que no cancro da mama o EGF não apresenta altos níveis de expressão quando co-expresso com EGFR e HER2, relativamente a outros ligandos, embora seja expresso no tumor [Révillion *et al.*, 2007]. Adicionalmente, no cancro da mama são descritas formas constitutivamente activas de EGFR [Mosessan *et al.*, 2004; Roskoski, 2004].

4.1.2 Polimorfismo A61G no Gene *EGF* e Cancro do Colo do Útero

No carcinoma do colo do útero, as mulheres mais novas (< 47 anos), com doença avançada e portadoras do genótipo G apresentam um aumento de risco para desenvolver esta neoplasia (OR=3,17; p=0,016).

O cancro do colo do útero está fortemente associado com o vírus HPV, sendo aceite que os tipos de alto risco são detectados em quase 100% de todos os cancros cervicais [Bekkers *et al.*, 2004]. No cancro cervical ocorre uma gradativa progressão, associada a anomalias citológicas, que culminam no carcinoma. Contudo, a presença das infecções com HPV de alto risco são necessárias, mas não suficientes, para promover o desenvolvimento deste carcinoma [Trottier *et al.*, 2006]. A persistência da infecção por HPV é intercedida por diversos factores, como: uso de contraceptivos orais, paridade, outras doenças sexualmente transmitidas, tabagismo, nutrição, e o *background* genético de cada indivíduo [Trottier *et al.*, 2006]. Actualmente aceita-se que a variabilidade genética desempenha um papel significativo na predisposição para cancro cervical.

As vias activadas pelo EGFR contribuem para a proliferação, apoptose, angiogénese e mestatização, estando envolvidas no desenvolvimento e progressão para cancro [Ciardiello *et al.*, 2001]. Adicionalmente, é proposto que a sobre-expressão do EGFR conduz à aquisição da capacidade poliferativa e progressão tumoral, por parte das células tumorais [Johnson *et al.*, 2004], sendo sugerido que a co-expressão do EGFR e os seus ligandos estão associados a um pior prognóstico de sobrevivência [Holbro *et al.*, 2004], Nas displasias cervicais a expressão EGFR é detectada em 85-100% dos casos [Salomon *et al.*, 1995]. Adicionalmente no cancro cervical, é demonstrado um aumento da sinalização mediada por EGF em células que expressam a proteína E5 [Straight *et al.*, 1993; Venuti *et al.*, 1998; Suprynowicz *et al.*, 2008]. Straight *et al.* (1993). Também se demonstrou que os ceratinócitos que expressam a proteína E5, os altos níveis de EGFR devem-se a um aparente atraso na internalização e degradação do receptor, ocorrendo um maior número de receptores reciclados que voltam à superfície da célula, assim como uma maior magnitude na estimulação de sinal [Straight *et al.*, 1993]. Recentemente, Chiang *et al.*, (2007) demonstraram que o EGF está envolvido na activação da invasão celular através da regulação ou estimulação do NHE I (*Na+/H+ exchanger isoform I*), no cancro cervical. Estes factos podem explicar a associação do referido polimorfismo em *EGF* para aumento de risco, no subgrupo com cancro cervical de mulheres mais novas com doença avançada.

No que concerne à associação encontrada para mulheres mais novas, e com cancro avançado, é neste grupo de mulheres mais jovens que ocorre maior prevalência de infecção por HPV [Goldhaber-Fiebert *et al.*, 2007; Steben *et al.*, 2007; Kjaer *et al.*, 2008]. Pode-se colocar a hipótese que nas mulheres portadoras do alelo G existe uma pressão selectiva, fomentada pelos efeitos oncogénicos do HPV, favorável neste *background* genético. Na associação aos estadios avançados, o estudo de Gaffeney *et al.* (2003) demonstraram que pacientes com expressão de EGFR têm pior prognóstico na análise de sobrevivência, estando este receptor mais expresso nos estadios mais avançados, revelando-se consistente com os resultados apresentados.

4.2 Conclusões Finais

O EGF é um factor de crescimento envolvido na activação da transdução de sinal para a proliferação celular, através da interacção com o EGFR. Alterações nestas vias, que promovem um ganho de função, estão envolvidas na promoção de neoplasias. O polimorfismo A61G do gene *EGF* está, em situações particulares, associado com a patologia neoplásica, nomeadamente melanomas e cancro gástrico, embora exista ainda pouca consistência entre estudos.

Este trabalho foi efectuado em três carcinomas, ovário, mama e colo do útero, tendo cada um deles resultados distintos. No cancro do ovário os portadores da variação 61G* apresentam maior protecção para desenvolvimento da doença, assim como desenvolvem-na mais tarde. Este efeito protector também é encontrado nos indivíduos diagnosticados com doença avançada, assim como nas mulheres mais novas que 53 anos. Este aparente paradoxo poderá ser explicado pela internalização e degradação do EGFR como resultado dos mecanismos de regulação do mesmo. No cancro da mama os resultados são também consistentes com a hipótese da internalização e degradação do EGFR aquando da ligação do EGF, permitindo explicar um decréscimo no risco para desenvolver cancro da mama para os portadores do alelo G, assim como o de desenvolverem a doença mais tarde.

No cancro do colo do útero, embora o HPV seja o principal factor de risco, é aceite a influência do *background* genético, nomeadamente a associação com risco em mulheres mais novas e mulheres com doença avançada portadoras do alelo G, podendo ser explicados pelo facto das mais novas terem maior prevalência de infecção pelo HPV, assim como a activação do EGF às vias de sinalização está associado a pior prognóstico nos pacientes com cancro cervical.

Deste modo é possível que cada órgão ou tecido tenha um determinado micro-ambiente em volta do EGFR, ocorrendo diferentes mecanismos de regulação deste receptor. Deste modo, podem ser explicados os diferentes resultados encontrados para os diferentes órgãos.

Adicionalmente, sendo o cancro uma doença multifactorial, é necessário considerar a influência de outros genes, em particular, os associados à promoção tumoral, que poderão auxiliar a compreensão dos factores envolvidos nesta complexa rede de processos. Deste modo, é possível uma integração dos resultados facilitando a interpretação dos mesmos.

Nas vias de activação da proliferação é necessário ter em conta que estão envolvidos diversos mecanismos de regulação, promovendo diferentes comportamentos das células conforme o ambiente circundante.

4.3 Considerações Finais

A ligação do EGF (família de ligandos) aos receptores ErbB promove a activação de uma audaz *network* nas vias de sinalização [Yarden *et al.*, 2001]. Devido à sua inerente complexidade, torna-se promissor conhecer o seu funcionamento, nomeadamente a configuração das interacções na teia de sinais e o tipo de envolvimento; nas quais participam a modularidade, redundância e controle das vias; através de sinais biológicos robustos que usam uma perfeita e audaz engenharia de sistemas [Citri *et al.*, 2006]. Estes processos são controlados por sinais *positivos* ou *negativos*, ocorrendo entre eles a degradação lisossomal de receptores para a atenuação de sinais [Yarden *et al.*, 2001; Citri *et al.*, 2006]. A associação do polimorfismo A61G para protecção no cancro do ovário e da mama pode ser explicada a este nível.

Contudo a *network* activada por ErbB pode ser integrada numa outra *network* que activa sinais usando hormonas, neurotransmissores, linfocinas e indutores de stresse [Yarden *et al.*, 2001]. Adicionalmente, cada órgão possui um característico micro-ambiente que o envolve, sendo de notar que no cancro da mama e do ovário, ambos sujeitos a um forte controle hormonal, observou-se uma associação para protecção; enquanto no cancro do colo do útero, de origem viral, observou-se associação para risco.

4.4 Perspectivas Futuras

De acordo com os resultados apresentados, pode-se concluir como perspectivas futuras as seguintes linhas de estudo:

-proceder ao aumento dos números da amostra em todos os modelos tumorais para completar este estudo, conferindo-lhe maior significância estatística.

-estender o estudo a outros modelos oncológicos, com o intuito de contribuir para o esclarecimento da importância deste polimorfismo como marcador molecular de susceptibilidade para cancro ou para doença avançada.

-estudo farmacogenómico, integrando dados sobre o tratamento dos pacientes, nomeadamente o tempo livre de doença e a sobrevida. Esta informação irá permitir correlacionar a resposta individual ao tratamento com o polimorfismo em estudo.

-estudos paralelos sobre quantificação da expressão do gene *EGF* e dos seus receptores ErbB activados, nos diferentes tumores, ajudariam na compreensão do mecanismo de actuação deste ligando.

-em linhas celulares adicionar genes repórter aos genes dos receptores *ErbB* e depois analisar o seu tráfego na célula, de diferentes tumores inclusivé dos diferentes tipos histológicos de cada tumor.

-aos estudos realizados com linhas celulares, adicionar a análise do genótipo do polimorfismo em estudo e verificar se existem diferenças nos parâmetros a serem analisados entre os diferentes genótipos.

Assim, toda a análise efectuada tentou contribuir para uma melhor compreensão deste polimorfismo na associação para cancro. Esta análise terá de se agrupar a muitas outras, em diferentes genes associados a cancro, nomeadamente polimorfismos noutros ligandos e no EGFR, que poderão ajudar a definir as características genéticas individuais para susceptibilidade à doença neoplásica. O conhecimento do *background* genético individual irá permitir definir a predisposição para cancro. Este conhecimento poderá ser utilizado na prevenção, ou ao nível de um tratamento clínico mais adequado a cada indivíduo.

Assim, num futuro próximo, o tratamento oncológico poderá ser baseado no *background* genético, onde cada indivíduo terá uma terapia individualizada.

5. REFERÊNCIAS BIBLIOGRÁFICAS

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6. ANEXOS

6.1 Anexo I

Provas do artigo intitulado *"Ovarian cancer and genetic susceptibility: association of A61G polymorphism in the EGF gene"*, aceite para publicação na revista *Experimental Biology and Medicine*.

Ovarian cancer and genetic susceptibility: association of A61G polymorphism in the *EGF* gene

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Deolinda Pereira[d], Daniela Pinto[a,e], Berta Sousa[d], Raquel Catarino[a], Rui Medeiros[a,c,e]

[a]Molecular Oncology Group, Portuguese Institute of Oncology, Porto Centre, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Medical Oncology Department, Portuguese Institute of Oncology, Porto Centre, Portugal

[e]Virology Department, Portuguese Institute of Oncology, Porto Centre, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Growth factors play an essential role in regulating cellular proliferation, and lack of control is characteristic of malignant development. The epidermal growth factor (*EGF*) gene codifies a growth factor that binds to the EGF receptor (EGFR), which is involved in activating pathways that promote cellular proliferation, survival, migration and differentiation. The purpose of this study was to appraise the association between *EGF* gene A61G polymorphism with ovarian cancer susceptibility. A total of 564 DNA samples were analysed from 175 women with ovarian cancer and 389 women without cancer, through PCR-RFLP. We found a decreased risk for developing ovarian cancer in GG carriers compared to AA carriers (OR = 0.46, CI = 0.25-0.83, *P* = 0.010). The seemingly protective role in GG carriers was observed in women under 53 years of age (OR = 0.38, 0.16-0.86, *P* = 0.011) and in patients diagnosed with advanced stage disease (OR = 0.38, 0.18-0.81, *P* = 0.012). Allelic comparison evidenced similar results, with decreased risk for G allele. We further observed a linear trend for G allele in cancer risk. Moreover, we analysed the influence of genotypes in the time to onset of the disease and observed that GG carriers had ovarian cancer later than AA carriers (*P* = 0.035). We hypothesize that this polymorphism confers protection for ovarian cancer development.

Keywords: EGF Polymorphism, Ovarian Cancer, EGFR.

Introduction

Extracellular environment controls cell survival, and growth factors are one of the most important signallers in this process (1). Epidermal growth factor (EGF) is a key factor in promoting cell survival, and after binding to EGF receptor (EGFR) it causes activation of several signal transduction pathways (1), promoting cell proliferation, survival, migration and differentiation (2, 3).

Growth factors are important in initiating and maintaining neoplastic transformation (4). Tumour cells synthesize high levels of growth factors regulating their proliferation through autocrine and paracrine mechanisms (2, 4). EGFR and its ligands are often overexpressed in human carcinomas and they contribute to tumour progression through different mechanisms (2). EGFR and EGF-like peptides are involved in over 70% of all cancers (5).

The reproductive tract malignancies have important repercussion in women. Ovarian cancer is the sixth most common cancer and the seventh cause of death from cancer in women, with approximately 204,000 new cases and 125,000 deaths in 2002 (6). The normal ovary expresses EGFR but when compared with primary ovarian carcinomas, the expression in this disease shows significantly higher levels (7). It has also been reported that EGF regulates oocyte maturation and follicular growth (8), and it is involved in autonomous proliferation of ovarian carcinoma cells (7).

Shahbazi et al. (9) identified a polymorphism at position 61 of the EGF gene consisting of a substitution of G for A (A61G). This variation in vitro produced less quantity of EGF in cells from AA individuals than cells from GG or GA individuals and consequently the GG carriers were associated with an increased risk of developing malignant melanoma

when compared with AA genotype ($P < 0.0001$) (9).To the best of our knowledge, no reports have been published regarding the role of EGF polymorphism in ovarian cancer. The aim of our study was to assess the effect of A61G polymorphism in ovarian cancer susceptibility in the Portuguese population.

Material and Methods

Subjects

This case-control study included 175 women (median 53 years of age) with histologically confirmed ovarian cancer, from the Portuguese Institute of Oncology-Porto between 1999 and 2001, according to other studies performed by our group (10, 11). Regarding the histological classification of tumours, 57% were serous, 15% clear cell, 14% mucinous, 10% endometrioid and 4% had other histological types. Patients were staged according to FIGO statements and were followed by the same medical oncologist. The cancer-free control women (n=389, median 41 years of age) were recruited from the Blood Donors Bank Institute. Individuals from both groups were Caucasian and residents in the same geographic area and all of them gave consent according to the Helsinki Declaration.

EGF A61G genotype analysis

Genomic DNA was extracted from 8 ml peripheral blood obtained with a standard venipuncture technique using EDTA-containing tubes, and isolated from the white blood cell fraction of each sample, using a standard salting out protocol (12). The EGF A61G genotypes were determined using the PCR-RFLP assay according to a previously

published protocol by Shahbazi et al. (9). Two primers, forward: 5'- TGT CAC TAA AGG AAA GGA GGT-3' and reverse: 5'- TTC ACA GAG TTT AAC AGC CC-3', were used to amplify the 242 base pair (bp) fragment. PCR reactions were performed in a 50µl reaction volume containing: 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0,3 µM of each primer and 1U Taq DNA polymerase. Thermocycler parameters were as follows: 95ºC for 5 min; 35 cycles of 94ºC for 60s, 55ºC for 60s and 72ºC for 60s; and a final extension step at 72ºC for 5 min. PCR products were digested overnight at 37ºC with 2 U of the restriction enzyme and 10 µl of PCR products to a final volume of 15 µl. The restriction fragments were then analyzed by electrophoresis in 3% (w/v) agarose gel stained with 0.5% ethidium bromide and photographed under UV illumination. The abrogation of the site recognized by the restriction enzyme AluI, produces 4 fragments of 15, 34, 91 and 102 bp (A allele), while the G allele corresponds to 3 fragments of 15, 34 and 193 bp. In the gel only fragments of 91, 102 and 193 bp are visible.

Statistical analysis

Chi-square test was used to compare categorical variables. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated to assess the relationship between the polymorphism and ovarian cancer susceptibility, followed by age-adjusted logistic regression analysis. Linear trend analysis was performed to evaluate the risk for cancer according to the presence of zero, one, or two G alleles. Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed vs. the expected genotype frequencies.

The waiting time to onset of the disease (WTO) was defined as the interval between the time of initial exposure to the risk factor (A61G) and the time of disease onset. Thus, we

calculated the cumulative probabilities for having disease by the Kaplan–Meier methodology and the primary analysis of time-to-event end points for WTO with the use of a two-sided log-rank test.

All statistical analysis in this study was performed with the computer software SPSS for Windows (version 15.0) and Epi Info (version 6.04), considering a statistical significant *P* value when $P < 0.05$.

Results

Genotype frequencies for both groups were: AA (0.38), AG (0.48) and GG (0.14) in ovarian cancer cases and AA (0.31), AG (0.46) and GG (0.23) for controls (Table1). Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium for both groups (controls, $P = 0.996$; cases, $P = 0.400$). The homozygous G carriers presented lower risk for developing ovarian cancer in crude (OR = 0.50, 95% CI = 0.28-0.89, $P = 0.011$) and in age-adjusted logistic regression analysis (OR = 0.46, 95% CI = 0.25-0.83, $P = 0.010$) (Table 1). Linear trend analysis showed a cumulative protective role effect for G allele in cancer susceptibility ($P = 0.014$).

Furthermore, women diagnosed before 53 years of age, who are simultaneously GG carriers, had protection for ovarian cancer (OR = 0.38, 95% CI = 0.16-0.86, $P = 0.011$) (Table2), although no association was found for women over 53 years old (data not shown). In stratified analysis according to stage of disease, also GG carriers were protected for being diagnosed with advanced stage (III and IV) (OR = 0.38, 95% CI = 0.18-0.81, $P = 0.012$) (Table2), albeit there was lack of association for earlier stages (data not shown).

Table 1: EGF genotype and allele frequencies in patients with ovarian cancer and in healthy controls.

	Cases (n=175) n (frequencies)	Controls (n=389) n (frequencies)	OR	95%CI	*P*
Genotype [a]					
AA	67 (0.38)	120 (0.31)	1		
AG	83 (0.48)	180 (0.46)	0.83	0.55-1.25	0.344
GG	25 (0.14)	89 (0.23)	**0.50** [b]	0.28-0.89	**0.011**
Allele					
A	217 (0.62)	420 (0.54)	1		
G	133 (0.38)	358 (0.46)	**0.72**	0.55-0.94	**0.012**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.014.

[b] Age adjusted risk for GG vs AA: OR=0.46, 95%CI=0.25-0.83, *P* =0.010.

Kaplan Meier cumulative hazard function plots and log-rank test showed an earlier onset of disease for homozygous A carriers when compared to homozygous G carriers (*P* = 0.035) (Fig. 1). We observed a trend for G allele cumulative protective effect in the WTO when all three genotypes were analysed (AA, AG and GG), although no statistically significant difference was achieved (median: AA, 62 years; AG, 65 years; GG, 70 years; *P* = 0.067).

Table 2: Association of A61G polymorphism with clinicopathological parameters in patients with ovarian cancer compared with controls.

	Cases	Controls			
	n (frequencies)	n (frequencies)	OR	95%CI	*P*
Age ≤53 (n=85)					
Genotype [a]					
AA	35 (0.41)	84 (0.29)	1		
AG	40 (0.47)	138 (0.49)	0.70	0.40-1.22	0.177
GG	10 (0.12)	64 (0.22)	**0.38**	0.16-0.86	**0.011**
Allele					
A	110 (0.65)	306 (0.53)	1		
G	60 (0.35)	266 (0.47)	**0.63**	0.43-0.91	**0.009**
FIGO stage III/IV[b] (n=97)					
Genotype [c]					
AA	40 (0.41)	120 (0.31)	1		
AG	46 (0.48)	180 (0.46)	0.77	0.46-1.28	0.280
GG	11 (0.11)	89 (0.23)	**0.37**[d]	0.17-0.80	**0.006**
Allele					
A	126 (0.65)	420 (0.54)	1		
G	68 (0.35)	358 (0.46)	**0.63**	0.45-0.89	**0.006**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.011.

[b] Staging for cases only.

[c] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.007.

[d] Age adjusted risk for GG *vs.* AA: OR=0.38, 95%CI=0.18-0.81, *P* = 0.012.

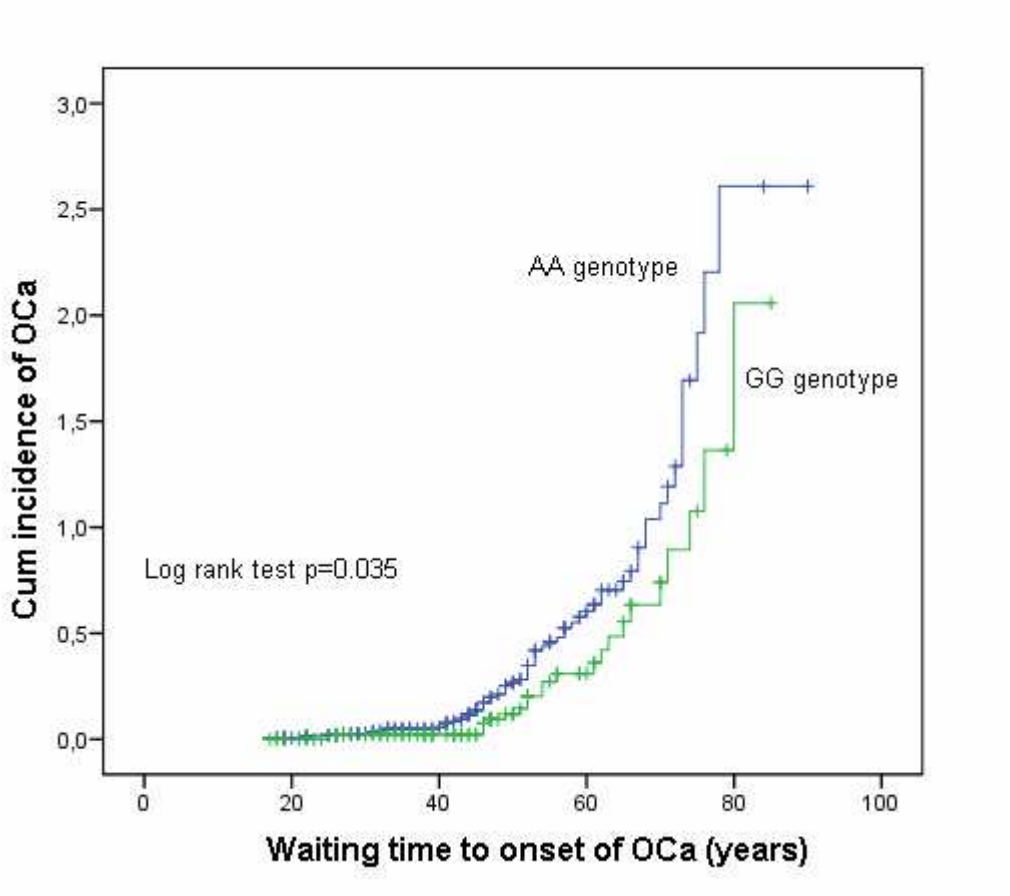

Figure. 1: Association between A61G polymorphism and the waiting time to onset of disease (WTO). Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test (P = 0.035).

Discussion

Cancer predisposition may be due to the combination of low penetrance genetic variants. Polymorphisms may have higher significance to public health than individual risks associated with familiar cancer (13–15). Genetic association studies on the *EGF* A61G genetic polymorphism have already been performed in cancer, with controversial results. Nevertheless, considerable concordance exists in *in vitro* studies associating the *EGF* 61 G allele with EGF overexpression (9, 16–22).

In this case-control study, we analysed the association between a functional polymorphism (A61G) of the *EGF* gene and the risk for developing ovarian cancer. We found a protective effect for the development of ovarian cancer in homozygous G carriers (OR = 0.46, *P* = 0.010). The protective effect of GG carriers was also observed for women ≤ 53 years of age (OR = 0.38; *P* = 0.011) and for being diagnosed with advanced disease (stage III and IV) (OR = 0.38; *P* = 0.012). Furthermore, Kaplan Meier curve analysis showed later onset of disease for GG genotype carriers, compared to AA (*P* = 0.035).

The EGF is a growth factor that activates a signal transduction pathway promoting proliferation, migration and differentiation (23); nevertheless, some authors showed that, after binding to its receptor, they are both internalized, sourcing the formation of endosomes and ultimately degradation (23, 24). The protective effect for the EGF overexpression in G allele carriers that we observed in this study may be explained by the increased bioavailability of EGF and the subsequence removed of EGFR from the cell surface, due to its degradation. In fact, other studies showed that EGFR expression on cell surface decreases abruptly when a certain concentration of EGF is added to cell lines (25, 26). Specific EGF ligands promote a long lasting effect, inhibiting EGFR recycling (26). A low availability of EGFR will decrease the EGF/EGFR pathway activation and eventually protect ovarian cancer development. Another recent study reports that in some cell lines, EGF paradoxically inhibits proliferation in high concentrations and induces loss of adhesion, cell cycle arrest and apoptosis (27).

It has been suggested that EGFR has pleiotropic cell responses (23) that are regulated by signals when numerous negative regulatory mechanisms act, such as: availability of the

ligand to the receptor and terminal signal inactivation through receptor internalization and degradation (24). Furthermore, this regulation varies according to the arrangements of ligand-receptor engagement, tyrosine phosphorylation and subsequent receptor dimerization combinations, as well as, on the stage and context of cell type, and cell growth, different signalling transduction pathways can be activated (24). As active pathways are not known in each different tumour, we can explain our different results for this tumour type, because different regulatory mechanisms of EGFR may depend on tissue or tumour type.

It is known that EGF acts in the ovary and consequently EGFR expression is present since the functional initiation of this organ. When we analysed the genotype influence in the time to onset of disease, we observed that GG carriers developed cancer later than the homozygous A. It is known that germinative line genetic variants are part of the genetic background of individuals from *in utero* until death. This is even more relevant if we are in the presence of a functional polymorphism, as in the case of *EGF* A61G. In this case it will impact in the concentration of growth factor both locally and systemically, eventually leading to less EGFR in ovarian cells, and consequently lower activation of the EGF/EGFR cascade. We hypothesize that women who carry G homozygous genotype are more prone to later onset of disease (8 years later).

The protective effect of GG also occurred in individuals that have more advanced staging. Overexpression of EGFR is more frequent in the ovarian cancer staged at III and IV (Lassus *et al.*, 2002) (28), suggesting that this pathway is relevant for advanced ovarian cancer. Considering this evidence, we hypothesize that GG carriers, who have lower EGFR

at cell surface, may have an increased protection for advanced stage ovarian cancer. Further studies on EGFR expression according to *EGF* A61G in ovarian tumours are required to confirm this hypothesis.

We also showed that GG carriers in younger women (<53 years) have lower risk for developing ovarian cancer. EGF is expressed in the ovary (29, 30) and acts in the evolution of ovarian follicles (31–33). It inhibits the expression of hormones produced in the ovary (estrogen and progesterone) and decreases of FSH (follicle-stimulating hormone) (34–37). Since younger women are more exposed to sexual hormones and these are associated with a greater cell proliferation, a decrease of these hormones promoted by higher levels of EGF in GG carriers reduces the risk for ovarian cancer in pre-menopausal women.

In conclusion the polymorphism A61G in ovarian cancer was found to confer protection. Protection was also found for being diagnosed with higher stage and in women younger than 53 years. The waiting time to onset of ovarian cancer was longer in GG carriers. These apparent paradoxal results involving *EGF* in ovarian cancer protection are supported by recent findings of EGFR internalization and degradation, which may be regulated through ligand availability. Further studies on this issue should be undertaken to analyse the role of EGF/EGFR pathway in ovarian cancer and the role of genetic background in this mechanism.

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6.2 Anexo II

Provas do artigo intitulado *"Epidermal growth factor genetic variation, breast cancer risk and waiting time to onset of disease"*, submetido para publicação na revista *DNA and Cell Biology*, ainda que sujeito a correcções pela mesma.

Epidermal growth factor genetic variation, breast cancer risk and waiting time to onset of disease

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Daniela Pinto[a,d], Deolinda Pereira[e], Berta Sousa[e], Joaquina Mauricio[e], Carlos Lopes[c], Rui Medeiros[a,c,d]

[a]Molecular Oncology Group, Portuguese Institute of Oncology - Porto, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Virology Department, Portuguese Institute of Oncology - Porto, Portugal

[e]Medical Oncology Department, Portuguese Institute of Oncology - Porto, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Growth factors are important mediators of proliferation. Deregulation in growth factor mechanisms as well as in its receptors will contribute to cancer development. One of the most important is the epidermal growth factor (EGF) which is encoded by *EGF* gene. A functional polymorphism at position 61 (A/G) is associated with increased expression of EGF. Thus, we proposed to assess genotype frequencies in a case-control study and appraise their association to breast cancer risk. Using the polymerase chain reaction technique combined with restriction enzyme fragment length polymorphism (PCR-RFLP) we analysed DNA from 883 women (500 controls and 383 breast cancer). Our results suggested that carriers of G homozygous genotype had a lower risk for developing breast cancer (OR = 0.68; 95% CI, 0.46-1.01). Furthermore, we showed that the waiting time for onset of breast cancer in G homozygous patients for EGF genotypes (55 years) was significantly lower in comparison to A-allele carriers (59 years) (log-rank test: p=0.041). EGF is produced in mammary tissue and acts in the mammalian development. A lower risk for breast cancer in GG carriers might be explained through of EGF receptor (EGFR) internalization promoted by EGF.

Keywords: EGF polymorphism, breast cancer, EGFR.

Introduction

Breast cancer incidence has increased worldwide, being the most frequent cancer among women with nearly 1,15 million new cases in the years 2002 (Parkin *et al.*, 2005).

Under normal physiological conditions, few growth factors are found in plasma and have a regenerative injury role as a function (Goustin *et al.*, 1986). In cancer cells these factors are deregulated, and frequently more than one can be required for proliferation (Goustin *et al.*, 1986). Epidermal growth factor (EGF) is one of the most important extra-cellular signalling molecules and a key regulatory promoter of cell survival (Henson *et al.*, 2006).

EGF binds to its receptor (EGFR) activating intracellular signalling transduction pathways. These signals culminate in cell proliferation, migration and differentiation (Arteaga, 2001; Holbro *et al.*, 2003; Mosesson *et al.*, 2004; Roskoski *et al.*, 2004; Araújo *et al.*, 2007; Bublil *et al.*, 2007). EGFR and its ligands are highly expressed in tumour cells (Arteaga *et al.*, 2001) and it is suggested that co-expression of EGFR and its ligands is associated with poor survival (Holbro *et al.*, 2004). Furthermore, it is known that the proliferative capacity acquired by overexpression of EGFR may promote tumour progression (Johnson *et al.*, 2004). Other reports indicate that cancer progression through EGFR-mediated signals include mechanisms such as angiogenesis, metastatic spread and apoptosis inhibition (Ciardiello *et al.*, 2001). EGFR and ErbB-2 are frequently overexpressed in several tumours including breast, colorectal, ovarian and non-small cell lung cancer (Roskoski *et al.*, 2004). Additionally, EGF is expressed in human breast carcinomas and human breast lines (Salomon *et al.*, 1995).

Results from Shahbazi *et al.* (2002) showed that a polymorphism in the *EGF* gene results in a nucleotide A to G substitution at position 61.[14] According to these authors and others, G carriers have significantly higher EGF production in peripheral-blood mononuclear cell cultures, glioblastomas, and gliomas cell lines (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a).

To the best of our knowledge, no reports regarding *EGF* polymorphism and breast cancer risk have been published. Therefore, we investigated the association of *EGF* gene A61G polymorphism to breast cancer development.

Material and Methods

Subjects

This case-control study was performed in 883 women including 383 patients with histologically diagnosed breast cancer followed at the Portuguese Institute of Oncology – Porto, and a control group of 500 women without cancer disease history. All participants provided informed consent and the individuals from both groups were from Caucasian ethnicity and were residents in the same geographic area.

Regarding breast cancer patients, the median age at diagnosis was 44 years (mean 46.9, standard deviation 14.1). Concerning histological classification, 89.0% of cases were invasive ductal carcinomas, 4.2% invasive lobular carcinomas and 6.8% consisted of other types (medullary carcinoma; papillary/micropapillary carcinoma; ductal carcinoma in situ; mucinous carcinoma and mixed (ductal and lobular; ductal and papillary)). Patients were

assessed according to the staging system and were followed by the same medical oncologist.

The healthy individuals for the control group were randomly recruited from the Institute's Blood Donor's Bank and had no evidence of neoplastic disease. The median age was 41 years with a mean of 41.7 and 14.7 of standard deviation.

DNA was extracted from peripheral blood samples obtained with a standard venipuncture technique using EDTA-containing tubes, according to previously published studies from our group (Medeiros *et al.*, 2004; Costa *et al.*, 2007b; Costa *et al.*, 2008).

EGF +61 A>G genotype analysis

The *+61 A>G* polymorphism (rs 4444903) was genotyped by the method previously reported by Shahbazi *et al.* (2002) using the PCR-RFLP technique. Amplification was carried out in a 50 µl reaction mixture containing: 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.3 µM of each primer and 1U Taq DNA polymerase. The cycling conditions comprised a hot start at 95ºC for 5 min, followed by 35 amplification cycles at 94ºC for 60s, 55ºC for 60s and 72ºC for 60s, followed by one elongation step at 72ºC for 5 min. A 242 base pair (bp) fragment was amplified using primers: F-5' TGT CAC TAA AGG AAA GGA GGT3' and R-5'TTC ACA GAG TTT AAC AGC CC3'. The A61G variation was identified with the restriction enzyme *Alu*I. 2U restriction enzyme was added to 10 µl PCR products in a final volume of 15 µl. The incubation was performed at 37ºC overnight. The products were separated on 3% agarose gels with 0.5% ethidium bromide and photographed under UV illumination.

After the destruction of the recognition site by the restriction enzyme, the A allele produced 4 fragments: 15, 34, 91 and 102 bp, while the G allele produced 3 fragments: 15, 34 and 193 bp. In the gel only the fragments: 91, 102 and 193 bp were visible.

Statistical analysis

The computer software SPSS for windows (version 15.0) and Epi Info (version 6.04) were used for all statistical analyses. Statistical calculations were performed using χ^2 test being conducted to compare categorical variables, and a 5% level of significance was used in the analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to assess the relationship between the polymorphism and breast cancer. The Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed vs. the expected genotype frequencies.

Furthermore, we considered the waiting time for the disease onset (WTO) as the interval between the time of initial exposure to the risk factor (*+61 A>G*) and the time of disease onset, as reported earlier (Costa *et al.*, 2007b; Costa *et al.*, 2008; Medeiros *et al.*, 2004). Thus, we calculated the cumulative probabilities for having disease by the Kaplan–Meier methodology and the primary analysis of time-to-event end points for WTO performed with the use of a two-sided log-rank test at the 5% level of significance.

Results

EGF genotype frequencies in breast cancer patients and control group are presented in Table 1. Frequencies of the genotypes were 29.8% for AA, 47.2% for AG and 23.0% for

GG in the control group and, 34.7% for AA, 47.0% for AG and 18.3% for GG in the breast cancer group. Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium for control group (p = 0.514). We found that GG carriers present a lower risk for developing breast cancer than AA carriers (OR = 0.68; 95% CI, 0.46-1.01; p = 0.047). Allelic frequencies were also significantly higher for G-allele carriers in controls compared to breast cancer patients (46.6% vs. 41.8%, respectively; p = 0.043) and a lower risk for breast cancer was observed in G allele carriers (OR = 0.82; 95% CI, 0.08-1.00) (Table 1). Furthermore, the analysis for the G allele in cancer susceptibility after stratification for age regarding the median age at diagnosis (44 years) demonstrate no statistically significant difference in the younger group (under 44 years) or in the older group (p=0.637 and p=0.591, respectively). Moreover, no difference were observed after stratification for stage (p=0.462).

Table 1 EGF genotype and allele frequencies in patients with breast cancer and in healthy controls.

	Cases (n=383)		Controls (n=500)		OR	95% CI	p
	n	%	n	%			
Genotypes [a]							
AA	133	34.7	149	29.8	1		
AG	180	47.0	236	47.2	0.85	0.62-1.17	0.310
GG	70	18.3	115	23.0	**0.68**	0.46-1.01	**0.047**
Alleles							
A allele	446	58.2	534	53.4			
G allele	320	41.8	466	46.6	**0.82**	0.08-1.00	**0.043**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: p = 0.048.

The cumulative probability of having an earlier breast cancer diagnosis (mean WTO), according to the presence or absence of the GG genotype, was statistically significant (55 years for A allele carriers vs. 59 years for non-carriers; log-rank test: p=55 years for A allele carriers vs. 59 years for non-carriers; log-rank test: p=0.041; OR= 1.353; 95% CI, 1.012-1.808) (Fig. 1).

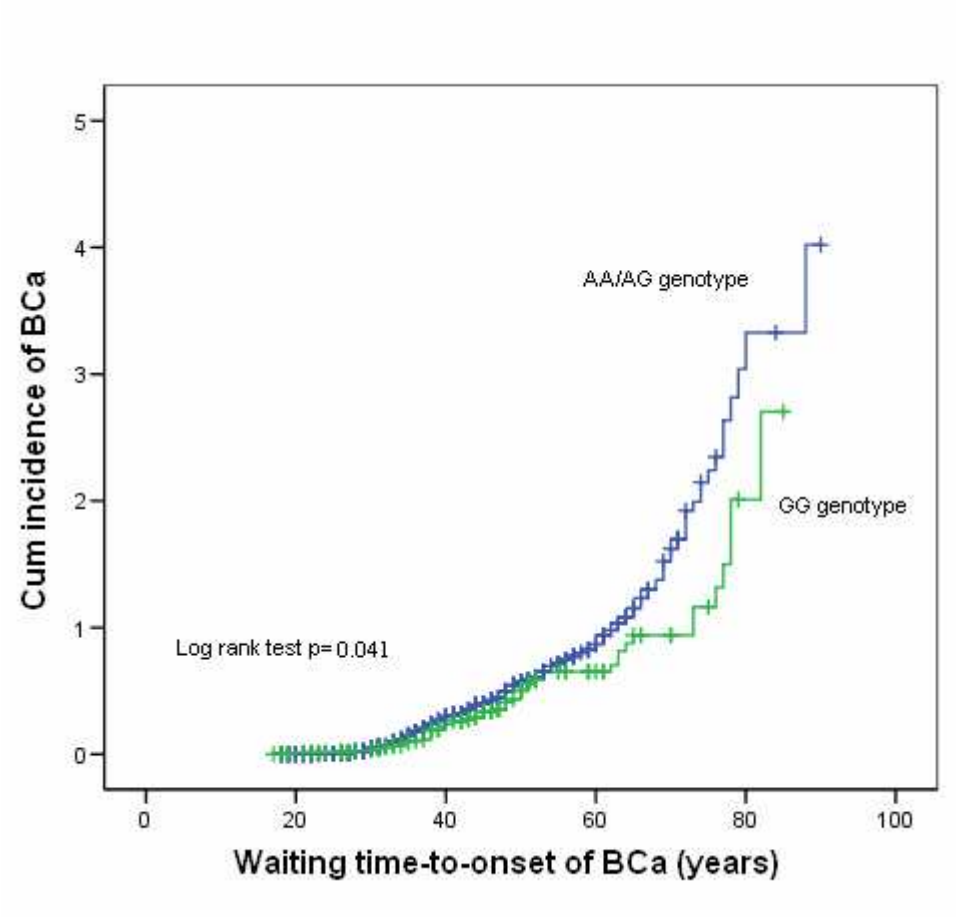

Fig.1 Association between A61G polymorphism and the waiting time to onset of disease (WTO). Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test (p = 0.041).

Discussion

EGF is a growth factor that activates a signalling transduction cascade responsible for activating genes involved in cell progression. The functional polymorphism *+61 A>G* in the *EGF* gene has been associated with increased risk for melanoma, glioma, gastric cancer, among others (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a; Rees *et al.*, 2002; McCarron *et al.*, 2003; Amend *et al.*, 2004; Hamai *et al.*, 2005; Ribeiro *et al.*, 2007), and it has been suggested that GG/AG carriers have higher production of EGF than AA carriers (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a) although no previous study has been published regarding the association of this polymorphism with breast cancer risk.

Our results demonstrated that *EGF* polymorphism may influence breast cancer risk. We observed that GG carriers have a lower risk for developing this neoplasia (OR=0.68; 95% CI, 0.46-1.01). Furthermore, allelic frequencies distribution suggest a protective role for breast cancer in G alleles carriers (OR = 0.82; 95% CI, 0.08-1.00). Moreover, we demonstrated a later onset of breast cancer in GG carriers (log-rank test: p=0.041). Our results must be observed with caution. In this study mean age of controls is lower than the mean age for the case group and age is a well know risk factor for cancer development. Further studies must include the adjustement of results for risk factors (age, smoking, alcohol drinking habits) and the information regarding survival may be a valuable tool for the understanding of the role of EGF in the follow up of breast cancer.

When compared with other ligands, EGF has distinct biological events and its actions are promoted by "down-regulation" of EGFR that will go to condition the different intracellular pathways activated (Taketani *et al.*, 1983). In fact, a plausible biological explanation for our results may be based upon a recently established mechanism for EGF-activation of EGFR internalization and degradation (Friedman *et al.*, 2005). According to this hypothesis, the EGF/EGFR complex is internalized through endossomal mechanism and as EGF is not sensible to acidic pH, dissociation does not occur, not allowing receptor recycling to the membrane surface (Wells *et al.*, 1999; Singh; *et al.*, 2005). It is interesting to observe that the EGFR is not internalized either if the ligand is TGF-α (Wells *et al.*, 1999; Singh; *et al.*, 2005) or if the ligand is EGF and its receptor are associated with ErbB-2 or ErbB-3 (Lenferink *et al.*, 1998). Furthermore, other studies demonstrate an abrupt decrease at surface expression of EGFR when cell lines are exposed to high levels EGF (Salazar *et al.*, 2002; Friedman *et al.*, 2005). Zhao *et al.* (2006) recently demonstrated that a high concentration of EGF induced loss of adhesion, cell cycle arrest, apoptosis, and inhibition of proliferation in cell lines. Thus, the putative higher EGF production of GG carriers may be consistent with breast cancer protection.

Another possible explanation could be the involvement of EGF in mammalian development (Taketani *et al.*, 1983; Fisher *et al.*, 1990; Spitzer *et al.*, 1995; Rosfjord *et al.*, 1999; Dehnhard *et al.*, 2000), and the ductal and alveolar epithelium stimulation (Fisher *et al.*, 1990; Spitzer *et al.*, 1995). EGF is localized in the inner layers of the terminal end bud and in ductal cell of mammary epithelium (Snedeker *et al.*, 1991). Also, EGF is present in the milk, being secreted by the mammary gland tissue during lactation (Fisher *et al.*, 1990), and with high concentrations being found inclusively after weaning (Fisher *et al.*, 1990; Beardmore *et al.*,1983; Moran *et al.*, 1983; Brown *et al.*, 1989). Interestingly, it was

suggested that breast cancer risk decreases with breast feeding, with having a child under the age of 24 and with an additional pregnancy (Russo *et al.*, 2005).

Results from the present study are consistent with the hypothesis of EGFR internalization in association with higher available EGF levels in breast cancer. A decrease in breast cancer development risk may be explained by internalization of EGF receptor (EGFR) promoted by EGF. Further studies may explore the role of EGF in definition of chemoprevention strategies or its meaning in therapeutic response of breast cancer.

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6.3 Anexo III

Provas do artigo intitulado *"Epidermal growth factor genetic variation and advanced cervical cancer in younger women"*, submetido para publicação na revista *Cancer Genetics and Cytogenetics*.

Epidermal growth factor genetic variation and advanced cervical cancer in younger women

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Deolinda Pereira[d], Daniela Pinto[a,c,e], Raquel Catarino[a,c], Rui Medeiros[a,c,e,f]

[a]Molecular Oncology Group, Portuguese Institute of Oncology - Porto, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Medical Oncology Department, Portuguese Institute of Oncology - Porto, Portugal

[e]Virology Department, Portuguese Institute of Oncology - Porto, Portugal

[f]Faculty of Health Sciences of Fernando Pessoa University, Porto, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Epidermal growth factor (EGF) stimulates cells proliferation through binding to its receptor (EGFR) and the overerexpression of this receptor is associated with a poorer prognosis. The *EGF* gene presents a polymorphism at position 61 (A/G), associated with higher EGF production. We examined the association between this polymorphism and cervical cancer through a case-control study. It was determined by the PCR-RFLP method in 384 women with cervical lesions and 500 controls from Caucasian ethnicity. Regarding patients with cervical cancer, we found an increase in the risk of advanced disease (FIGO stage IIb/IV) in younger G carriers (OR=3,17; 95% CI=1.214-8.257, p=0.016). We may hypothesize the onset of an advanced disease driven selective pressure due to the effect of oncogenic HPV types in a favourable genetic background observed in G carriers women. Present results suggest that EGF functional polymorphism may influence cervical cancer prognosis through EGF/EGFR pathway.

Keywords: EGF, polymorphism, cervical cancer, EGFR

Introduction

EGF is a growth factor discovered by Cohen (1962) [1] from the purified extracts of the salivary gland and then was associated with the function of stimulating the proliferation of epidermal tissues [2], after the binding to epidermal growth factor receptor (EGFR) [3]. Gray et al., 1983 showed that EGF is a polypeptide with 53 amino acids but it is synthesized from a large protein precursor of 1168 amino acids [4], and Morton *et al.* (1986) located the gene in locus 4q 25-27 [5].

It is known that the EGFR is a pleiotropic signaller that activates pathways involved in cell proliferation, migration and differentiation [6]. EGF binds to its receptor and induces a signalling cascade that culminates in cell transcription [7, 8]. This signal transduction also occurs in cancer cells [9] and tumours with overexpression of EGFR are strongly associated with a poor prognosis [10]. Because the EGFR are associated to bad prognosis in tumour progression fate is necessary to implement the new therapeutic targets and network strategies [11, 12]. Cervical cancer is the second most common cancer in women, with 493,000 estimated new cases and 274,000 deaths in the year 2002 [13] and the majority of cervical carcinomas express high levels of the EGFR [14].

Shahbazi *et al.* (2002) identified a functional polymorphism at position 61 of the *EGF* gene, which consists on a substitution of adenine (A) for guanine (G). AA genotype carriers have lower levels of EGF expression than individuals with the GG or AG genotypes [15].

In this case-control study we analysed this polymorphism in association with the risk of developing cervical cancer in a high incidence Caucasian population.

Material and Methods

Subjects

In this case control study, 884 women were genotyped for the *EGF* A61G polymorphism. Three-hundred and eighty four (384) had cervical lesions (that included 233 with invasive cervical carcinoma (ICC)), and 500 had non-cancer controls. The patients were recruited at the Portuguese Institute of Oncology, Porto centre, between 1999 and 2004, according to previously reported studies [16-20]. The median age at diagnosis for women with squamous intraepithelial lesions (SIL) was 35 years and 47 for women with ICC. In the group of patients with ICC 78.4% had squamous histologcal type, 12.4% adenocarcinoma and 3.9% other types. Tumour staging was assessed according to the FIGO staging system and were followed by the same medical oncologist. Individuals from the control group did not report clinical or pathological cervical disease and were recruited from the Institute's Blood Donors Bank, with 41 years of median age. All participants provided informed consent according to the Helsinki Declaration and the individuals from both groups were from Caucasian ethnicity and were residents in the same geographic area.

EGF A61G genotype analysis

Genomic DNA was extracted from 8ml of blood obtained with a standard venipuncture technique using EDTA-containing tubes of which was utilized the white blood cell fraction of each sample, using a standard salting out protocol [21].

The EGF A61G polymorphism was analyzed by PCR-RFLP essay using two primers (F-5' TGT CAC TAA AGG AAA GGA GGT3' and R-5'TTC ACA GAG TTT AAC AGC CC3') as described previously by Shahbazi *et al.* (2002) [15]. The PCR reaction was

carried out in a 50µl mixture containing 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.3 µM of each primer and 1U Taq DNA polymerase. These reaction mixtures were heated at 95ºC for 5 min; 35 cycles at 94ºC for 60 s, 55ºC for 60 s and 72ºC for 60 s; and a final extension step at 72ºC for 5 min. 10 µl PCR products were digested overnight at 37ºC with 2 U *Alu*I restriction enzyme to a final volume of 15 µl. The restriction fragments were then analyzed by electrophoresis in 3% (w/v) agarose gels stained with 0.5% ethidium bromide and photographed under UV illumination.

Statistical analysis

SPSS for windows (version 15.0) and Epi Info (version 6.04) were used for all statistical analyses. Statistical calculations were performed using χ^2 test in categorical variables. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to assess the association of the genetic variant with cervical disease and/or cancer. The Hardy-Weinberg equilibrium was tested by a Pearson of goodness fit test to compare the observed vs. the expected genotype frequencies.

Results

The frequencies of EGF genotypes in the cervical lesions group and the control group are exhibited in table 1 and 2. The frequencies of the genotypes were 29.8% for AA and 70.2% for AG/GG in the control group, 28.9% for AA and 71.1% for AG/GG in the entire group with cervical lesions and 27.0% for AA and 73.0% for AG/GG in the group with ICC. Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium

for both groups (controls: p = 0,514; cases: p = 0,978). No significant differences were found in subjects who carried G lack of increased risk of developing cervical lesions (p=0.773), and ICC (p=0.443), as shown in Table 1.

Table1: Association of A61G polymorphism with clinicopathological parameters in patients with cervical lesions

	Cases		Controls		OR	95%CI	p
	n	%	n	%			
Cervical lesions							
Genotypes							
AA	111	28.9	149	29.8	1		
AG	189	49.2	236	47.2	1.08	0.78-1.49	0.649
GG	84	21.9	115	23.0	0.98	0.66-1.45	0.918
AG+GG	273	71.1	351	70.2	1.04	0.78-1.40	0.773
ICC							
Genotypes							
AA	63	27.0	149	29.8	1		
AG	117	50.2	236	47.2	1.17	0.80-1.72	0.397
GG	53	22.8	115	23.0	1.09	0.69-1.73	0.700
AG+GG	170	73.0	351	70.2	1.15	0.81-1.62	0.443

When we made adjustments concerning age, histology and staging, we found an increased risk of G carriers genotypes with stage IIb-IV (considered as advanced disease) and younger than 47 years were associated with an advanced cancer (OR, 3.166; 95% CI, 1.214- 8.257; p=0.016) (Table 2). For other characteristics no associations were found (Table 1 and Table 2).

Table2: Association for advanced disease[a)] in G carriers (A61G polymorphism) in women under the age of 47 years

	p	OR	95%CI
Advanced disease[a)]	0.064	1.89	0.96-3.70
Advanced disease[a)] with age ≤ 47	**0,016**	**3.17**	**1.21-8.26**
Advanced disease[a)] with age > 47	0,520	0.69	0.22-2.15

[a)]Advanced disease: FIGO stage IIb/IV (reference localized disease: stage I/IIa)

Discussion

Cervical cancer has a straight reasoning linking the disease with the genital human papillomavirus (HPV), considering that the high-risk HPV (hr-HPV) genotypes are detected in almost 100% of all cervical cancers [23]. Cervical cancer develops through a continuum of progression of the different degrees with cytological abnormalities culminating in carcinoma. However, the presence hr-HPV infections are necessary, but not sufficient, to be the cause of this carcinoma [24]. The persistence of HPV infection is mediated by several factors - oral contraceptive use, parity, other STIs (Sexually Transmitted Infections), smoking, nutrition and host genetics [24]. Currently it was shown that genetic variability might play a significant role in cervical cancer predisposition [16-19, 25-28].

The objective present study was to examine the relationship between a functional EGF polymorphism (A61G) and cervical cancer. Results showed an increased risk for advanced disease in younger women (OR=3.17). This result may be explained through the higher production of EGF in G carries, which might increase cell proliferation signalling. Other

studies in melanoma, glioma, gastric cancer, observed that this polymorphism was associated with an increased risk for cancer development [15, 19, 30-35]. Additionally, a previous study failed to demonstrate cervical cancer risk association with EGF genotypes in East Asian individuals [36]. To the best of our knowledge, we present the first study in Caucasian women regarding the cervical cancer risk and EGF polymorphism. A study by Kang *et al.* (2007) of Korean patients revealed increased tendency of lymph node in G carriers [36]. Our results are consistent with this report however we observed that younger women simultaneously G carriers had increased risk of advanced cervical cancer (Table 2).

It is known that the EGFR pathways contribute to cell proliferation, apoptosis, angiogenesis and metastatic spread. All these processes are involved in cancer development and progression [37]. It has been suggested that EGFR overexpression might drive to the acquisition of proliferative capacity and tumour progression by tumour cells [38]. Moreover, earlier clinical reports suggest that coexpression of EGFR and its ligands (including EGF) are associated with poor survival of patients [12] and in cervical dysplasias 85-100% can be detected EGFR expression [14]. Also in cervical cancer, it was showed that EGF signalling is enhanced in E5-expressing cells [39-41]. Straight et al. (1993) demonstrated that the levels of EGFR are higher in E5-expressing keratinocytes cells because exist an apparent delay in the internalization and degradation of this receptor, and a greater number of receptors return to the cell surface than control keratinocytes, as well as a greater magnitude of ligand-stimulated phosphorylation of the EGFR tyrosine residue [39]. Recently, a report from Chiang *et al.* (2007) demonstrated that EGF is involved in the activation of cell invasion through upregulation or stimulation of NHE I

(Na+/H+ exchanger isoform I), in cervical cancer cells [42].This facts can explain our association between EGF and cervical cancer.

Concerning our observation of an increased advanced cancer the risk associated with women younger than 47 years we may suggest an association with the prevalence of HPV infection (low-risk or high-risk) to be relatively higher in this group when compared with older women [43-45]. We may hypothesize that it may exists an advanced disease driven selective pressure due to the effect of oncogenic HPV types in a favourable genetic background observed in G carriers women.

With concerning to association found for advanced stages, Gaffeney *et al.* 2003 demonstrated that patients with EGFR expression have less disease-free survival and overall survival, and concluded that the lower disease-free survival was higher as FIGO stages [47]. According to our results we suggest that the EGF/EGFR pathway only significantly impacts the risk for advanced ICC in younger women (< 47 years).

In conclusion the HPV is the first established risk factor in cervical cancer, although EGF A61G polymorphism may also influence this neoplasia. This fact only happens in women younger than 47 years and leads to advanced stages. This can be explained by younger women to have more HPV infection prevalence, and on the other hand, the action of EGF and its signalling are associated with poor prognosis in cervical cancer patients.

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6. ANEXOS

6.1 Anexo I

Provas do artigo intitulado *"Ovarian cancer and genetic susceptibility: association of A61G polymorphism in the EGF gene"*, aceite para publicação na revista *Experimental Biology and Medicine*.

Ovarian cancer and genetic susceptibility: association of A61G polymorphism in the *EGF* gene

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Deolinda Pereira[d], Daniela Pinto[a,e], Berta Sousa[d], Raquel Catarino[a], Rui Medeiros[a,c,e]

[a]Molecular Oncology Group, Portuguese Institute of Oncology, Porto Centre, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Medical Oncology Department, Portuguese Institute of Oncology, Porto Centre, Portugal

[e]Virology Department, Portuguese Institute of Oncology, Porto Centre, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Growth factors play an essential role in regulating cellular proliferation, and lack of control is characteristic of malignant development. The epidermal growth factor (*EGF*) gene codifies a growth factor that binds to the EGF receptor (EGFR), which is involved in activating pathways that promote cellular proliferation, survival, migration and differentiation. The purpose of this study was to appraise the association between *EGF* gene A61G polymorphism with ovarian cancer susceptibility. A total of 564 DNA samples were analysed from 175 women with ovarian cancer and 389 women without cancer, through PCR-RFLP. We found a decreased risk for developing ovarian cancer in GG carriers compared to AA carriers (OR = 0.46, CI = 0.25-0.83, *P* = 0.010). The seemingly protective role in GG carriers was observed in women under 53 years of age (OR = 0.38, 0.16-0.86, *P* = 0.011) and in patients diagnosed with advanced stage disease (OR = 0.38, 0.18-0.81, *P* = 0.012). Allelic comparison evidenced similar results, with decreased risk for G allele. We further observed a linear trend for G allele in cancer risk. Moreover, we analysed the influence of genotypes in the time to onset of the disease and observed that GG carriers had ovarian cancer later than AA carriers (*P* = 0.035). We hypothesize that this polymorphism confers protection for ovarian cancer development.

Keywords: EGF Polymorphism, Ovarian Cancer, EGFR.

Introduction

Extracellular environment controls cell survival, and growth factors are one of the most important signallers in this process (1). Epidermal growth factor (EGF) is a key factor in promoting cell survival, and after binding to EGF receptor (EGFR) it causes activation of several signal transduction pathways (1), promoting cell proliferation, survival, migration and differentiation (2, 3).

Growth factors are important in initiating and maintaining neoplastic transformation (4). Tumour cells synthesize high levels of growth factors regulating their proliferation through autocrine and paracrine mechanisms (2, 4). EGFR and its ligands are often overexpressed in human carcinomas and they contribute to tumour progression through different mechanisms (2). EGFR and EGF-like peptides are involved in over 70% of all cancers (5).

The reproductive tract malignancies have important repercussion in women. Ovarian cancer is the sixth most common cancer and the seventh cause of death from cancer in women, with approximately 204,000 new cases and 125,000 deaths in 2002 (6). The normal ovary expresses EGFR but when compared with primary ovarian carcinomas, the expression in this disease shows significantly higher levels (7). It has also been reported that EGF regulates oocyte maturation and follicular growth (8), and it is involved in autonomous proliferation of ovarian carcinoma cells (7).

Shahbazi et al. (9) identified a polymorphism at position 61 of the EGF gene consisting of a substitution of G for A (A61G). This variation in vitro produced less quantity of EGF in cells from AA individuals than cells from GG or GA individuals and consequently the GG carriers were associated with an increased risk of developing malignant melanoma

when compared with AA genotype ($P < 0.0001$) (9).To the best of our knowledge, no reports have been published regarding the role of EGF polymorphism in ovarian cancer. The aim of our study was to assess the effect of A61G polymorphism in ovarian cancer susceptibility in the Portuguese population.

Material and Methods

Subjects

This case-control study included 175 women (median 53 years of age) with histologically confirmed ovarian cancer, from the Portuguese Institute of Oncology-Porto between 1999 and 2001, according to other studies performed by our group (10, 11). Regarding the histological classification of tumours, 57% were serous, 15% clear cell, 14% mucinous, 10% endometrioid and 4% had other histological types. Patients were staged according to FIGO statements and were followed by the same medical oncologist. The cancer-free control women (n=389, median 41 years of age) were recruited from the Blood Donors Bank Institute. Individuals from both groups were Caucasian and residents in the same geographic area and all of them gave consent according to the Helsinki Declaration.

EGF A61G genotype analysis

Genomic DNA was extracted from 8 ml peripheral blood obtained with a standard venipuncture technique using EDTA-containing tubes, and isolated from the white blood cell fraction of each sample, using a standard salting out protocol (12). The EGF A61G genotypes were determined using the PCR-RFLP assay according to a previously

published protocol by Shahbazi et al. (9). Two primers, forward: 5'- TGT CAC TAA AGG AAA GGA GGT-3' and reverse: 5'- TTC ACA GAG TTT AAC AGC CC-3', were used to amplify the 242 base pair (bp) fragment. PCR reactions were performed in a 50µl reaction volume containing: 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0,3 µM of each primer and 1U Taq DNA polymerase. Thermocycler parameters were as follows: 95ºC for 5 min; 35 cycles of 94ºC for 60s, 55ºC for 60s and 72ºC for 60s; and a final extension step at 72ºC for 5 min. PCR products were digested overnight at 37ºC with 2 U of the restriction enzyme and 10 µl of PCR products to a final volume of 15 µl. The restriction fragments were then analyzed by electrophoresis in 3% (w/v) agarose gel stained with 0.5% ethidium bromide and photographed under UV illumination. The abrogation of the site recognized by the restriction enzyme AluI, produces 4 fragments of 15, 34, 91 and 102 bp (A allele), while the G allele corresponds to 3 fragments of 15, 34 and 193 bp. In the gel only fragments of 91, 102 and 193 bp are visible.

Statistical analysis

Chi-square test was used to compare categorical variables. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated to assess the relationship between the polymorphism and ovarian cancer susceptibility, followed by age-adjusted logistic regression analysis. Linear trend analysis was performed to evaluate the risk for cancer according to the presence of zero, one, or two G alleles. Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed vs. the expected genotype frequencies.

The waiting time to onset of the disease (WTO) was defined as the interval between the time of initial exposure to the risk factor (A61G) and the time of disease onset. Thus, we

calculated the cumulative probabilities for having disease by the Kaplan–Meier methodology and the primary analysis of time-to-event end points for WTO with the use of a two-sided log-rank test.

All statistical analysis in this study was performed with the computer software SPSS for Windows (version 15.0) and Epi Info (version 6.04), considering a statistical significant *P* value when $P < 0.05$.

Results

Genotype frequencies for both groups were: AA (0.38), AG (0.48) and GG (0.14) in ovarian cancer cases and AA (0.31), AG (0.46) and GG (0.23) for controls (Table1). Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium for both groups (controls, $P = 0.996$; cases, $P = 0.400$). The homozygous G carriers presented lower risk for developing ovarian cancer in crude (OR = 0.50, 95% CI = 0.28-0.89, $P = 0.011$) and in age-adjusted logistic regression analysis (OR = 0.46, 95% CI = 0.25-0.83, $P = 0.010$) (Table 1). Linear trend analysis showed a cumulative protective role effect for G allele in cancer susceptibility ($P = 0.014$).

Furthermore, women diagnosed before 53 years of age, who are simultaneously GG carriers, had protection for ovarian cancer (OR = 0.38, 95% CI = 0.16-0.86, $P = 0.011$) (Table2), although no association was found for women over 53 years old (data not shown). In stratified analysis according to stage of disease, also GG carriers were protected for being diagnosed with advanced stage (III and IV) (OR = 0.38, 95% CI = 0.18-0.81, $P = 0.012$) (Table2), albeit there was lack of association for earlier stages (data not shown).

Table 1: EGF genotype and allele frequencies in patients with ovarian cancer and in healthy controls.

	Cases (n=175) n (frequencies)	Controls (n=389) n (frequencies)	OR	95%CI	*P*
Genotype [a]					
AA	67 (0.38)	120 (0.31)	1		
AG	83 (0.48)	180 (0.46)	0.83	0.55-1.25	0.344
GG	25 (0.14)	89 (0.23)	**0.50** [b]	0.28-0.89	**0.011**
Allele					
A	217 (0.62)	420 (0.54)	1		
G	133 (0.38)	358 (0.46)	**0.72**	0.55-0.94	**0.012**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.014.

[b] Age adjusted risk for GG vs AA: OR=0.46, 95%CI=0.25-0.83, *P* =0.010.

Kaplan Meier cumulative hazard function plots and log-rank test showed an earlier onset of disease for homozygous A carriers when compared to homozygous G carriers (*P* = 0.035) (Fig. 1). We observed a trend for G allele cumulative protective effect in the WTO when all three genotypes were analysed (AA, AG and GG), although no statistically significant difference was achieved (median: AA, 62 years; AG, 65 years; GG, 70 years; *P* = 0.067).

Table 2: Association of A61G polymorphism with clinicopathological parameters in patients with ovarian cancer compared with controls.

	Cases	Controls			
	n (frequencies)	n (frequencies)	OR	95%CI	*P*
Age ≤53 (n=85)					
Genotype [a]					
AA	35 (0.41)	84 (0.29)	1		
AG	40 (0.47)	138 (0.49)	0.70	0.40-1.22	0.177
GG	10 (0.12)	64 (0.22)	**0.38**	0.16-0.86	**0.011**
Allele					
A	110 (0.65)	306 (0.53)	1		
G	60 (0.35)	266 (0.47)	**0.63**	0.43-0.91	**0.009**
FIGO stage III/IV[b] (n=97)					
Genotype [c]					
AA	40 (0.41)	120 (0.31)	1		
AG	46 (0.48)	180 (0.46)	0.77	0.46-1.28	0.280
GG	11 (0.11)	89 (0.23)	**0.37**[d]	0.17-0.80	**0.006**
Allele					
A	126 (0.65)	420 (0.54)	1		
G	68 (0.35)	358 (0.46)	**0.63**	0.45-0.89	**0.006**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.011.

[b] Staging for cases only.

[c] Analysis for linear trend according to the presence of null, one, or two G alleles: *P* = 0.007.

[d] Age adjusted risk for GG *vs.* AA: OR=0.38, 95%CI=0.18-0.81, *P* = 0.012.

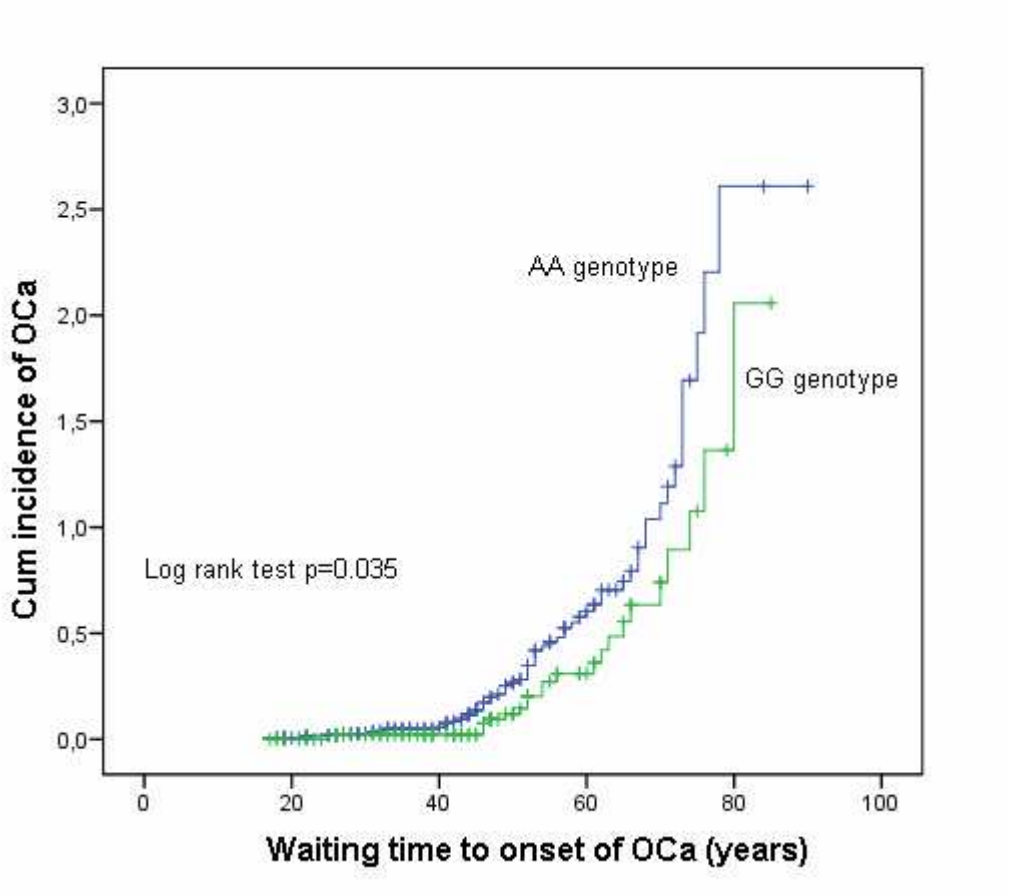

Figure. 1: Association between A61G polymorphism and the waiting time to onset of disease (WTO). Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test (P = 0.035).

Discussion

Cancer predisposition may be due to the combination of low penetrance genetic variants. Polymorphisms may have higher significance to public health than individual risks associated with familiar cancer (13–15). Genetic association studies on the *EGF* A61G genetic polymorphism have already been performed in cancer, with controversial results. Nevertheless, considerable concordance exists in *in vitro* studies associating the *EGF* 61 G allele with EGF overexpression (9, 16–22).

In this case-control study, we analysed the association between a functional polymorphism (A61G) of the *EGF* gene and the risk for developing ovarian cancer. We found a protective effect for the development of ovarian cancer in homozygous G carriers (OR = 0.46, *P* = 0.010). The protective effect of GG carriers was also observed for women ≤ 53 years of age (OR = 0.38; *P* = 0.011) and for being diagnosed with advanced disease (stage III and IV) (OR = 0.38; *P* = 0.012). Furthermore, Kaplan Meier curve analysis showed later onset of disease for GG genotype carriers, compared to AA (*P* = 0.035).

The EGF is a growth factor that activates a signal transduction pathway promoting proliferation, migration and differentiation (23); nevertheless, some authors showed that, after binding to its receptor, they are both internalized, sourcing the formation of endosomes and ultimately degradation (23, 24). The protective effect for the EGF overexpression in G allele carriers that we observed in this study may be explained by the increased bioavailability of EGF and the subsequence removed of EGFR from the cell surface, due to its degradation. In fact, other studies showed that EGFR expression on cell surface decreases abruptly when a certain concentration of EGF is added to cell lines (25, 26). Specific EGF ligands promote a long lasting effect, inhibiting EGFR recycling (26). A low availability of EGFR will decrease the EGF/EGFR pathway activation and eventually protect ovarian cancer development. Another recent study reports that in some cell lines, EGF paradoxically inhibits proliferation in high concentrations and induces loss of adhesion, cell cycle arrest and apoptosis (27).

It has been suggested that EGFR has pleiotropic cell responses (23) that are regulated by signals when numerous negative regulatory mechanisms act, such as: availability of the

ligand to the receptor and terminal signal inactivation through receptor internalization and degradation (24). Furthermore, this regulation varies according to the arrangements of ligand-receptor engagement, tyrosine phosphorylation and subsequent receptor dimerization combinations, as well as, on the stage and context of cell type, and cell growth, different signalling transduction pathways can be activated (24). As active pathways are not known in each different tumour, we can explain our different results for this tumour type, because different regulatory mechanisms of EGFR may depend on tissue or tumour type.

It is known that EGF acts in the ovary and consequently EGFR expression is present since the functional initiation of this organ. When we analysed the genotype influence in the time to onset of disease, we observed that GG carriers developed cancer later than the homozygous A. It is known that germinative line genetic variants are part of the genetic background of individuals from *in utero* until death. This is even more relevant if we are in the presence of a functional polymorphism, as in the case of *EGF* A61G. In this case it will impact in the concentration of growth factor both locally and systemically, eventually leading to less EGFR in ovarian cells, and consequently lower activation of the EGF/EGFR cascade. We hypothesize that women who carry G homozygous genotype are more prone to later onset of disease (8 years later).

The protective effect of GG also occurred in individuals that have more advanced staging. Overexpression of EGFR is more frequent in the ovarian cancer staged at III and IV (Lassus *et al.*, 2002) (28), suggesting that this pathway is relevant for advanced ovarian cancer. Considering this evidence, we hypothesize that GG carriers, who have lower EGFR

at cell surface, may have an increased protection for advanced stage ovarian cancer. Further studies on EGFR expression according to *EGF* A61G in ovarian tumours are required to confirm this hypothesis.

We also showed that GG carriers in younger women (<53 years) have lower risk for developing ovarian cancer. EGF is expressed in the ovary (29, 30) and acts in the evolution of ovarian follicles (31–33). It inhibits the expression of hormones produced in the ovary (estrogen and progesterone) and decreases of FSH (follicle-stimulating hormone) (34–37). Since younger women are more exposed to sexual hormones and these are associated with a greater cell proliferation, a decrease of these hormones promoted by higher levels of EGF in GG carriers reduces the risk for ovarian cancer in pre-menopausal women.

In conclusion the polymorphism A61G in ovarian cancer was found to confer protection. Protection was also found for being diagnosed with higher stage and in women younger than 53 years. The waiting time to onset of ovarian cancer was longer in GG carriers. These apparent paradoxal results involving *EGF* in ovarian cancer protection are supported by recent findings of EGFR internalization and degradation, which may be regulated through ligand availability. Further studies on this issue should be undertaken to analyse the role of EGF/EGFR pathway in ovarian cancer and the role of genetic background in this mechanism.

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6.2 Anexo II

Provas do artigo intitulado *"Epidermal growth factor genetic variation, breast cancer risk and waiting time to onset of disease"*, submetido para publicação na revista *DNA and Cell Biology*, ainda que sujeito a correcções pela mesma.

Epidermal growth factor genetic variation, breast cancer risk and waiting time to onset of disease

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Daniela Pinto[a,d], Deolinda Pereira[e], Berta Sousa[e], Joaquina Mauricio[e], Carlos Lopes[c], Rui Medeiros[a,c,d]

[a]Molecular Oncology Group, Portuguese Institute of Oncology - Porto, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Virology Department, Portuguese Institute of Oncology - Porto, Portugal

[e]Medical Oncology Department, Portuguese Institute of Oncology - Porto, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Growth factors are important mediators of proliferation. Deregulation in growth factor mechanisms as well as in its receptors will contribute to cancer development. One of the most important is the epidermal growth factor (EGF) which is encoded by *EGF* gene. A functional polymorphism at position 61 (A/G) is associated with increased expression of EGF. Thus, we proposed to assess genotype frequencies in a case-control study and appraise their association to breast cancer risk. Using the polymerase chain reaction technique combined with restriction enzyme fragment length polymorphism (PCR-RFLP) we analysed DNA from 883 women (500 controls and 383 breast cancer). Our results suggested that carriers of G homozygous genotype had a lower risk for developing breast cancer (OR = 0.68; 95% CI, 0.46-1.01). Furthermore, we showed that the waiting time for onset of breast cancer in G homozygous patients for EGF genotypes (55 years) was significantly lower in comparison to A-allele carriers (59 years) (log-rank test: p=0.041). EGF is produced in mammary tissue and acts in the mammalian development. A lower risk for breast cancer in GG carriers might be explained through of EGF receptor (EGFR) internalization promoted by EGF.

Keywords: EGF polymorphism, breast cancer, EGFR.

Introduction

Breast cancer incidence has increased worldwide, being the most frequent cancer among women with nearly 1,15 million new cases in the years 2002 (Parkin *et al.*, 2005).

Under normal physiological conditions, few growth factors are found in plasma and have a regenerative injury role as a function (Goustin *et al.*, 1986). In cancer cells these factors are deregulated, and frequently more than one can be required for proliferation (Goustin *et al.*, 1986). Epidermal growth factor (EGF) is one of the most important extra-cellular signalling molecules and a key regulatory promoter of cell survival (Henson *et al.*, 2006).

EGF binds to its receptor (EGFR) activating intracellular signalling transduction pathways. These signals culminate in cell proliferation, migration and differentiation (Arteaga, 2001; Holbro *et al.*, 2003; Mosesson *et al.*, 2004; Roskoski *et al.*, 2004; Araújo *et al.*, 2007; Bublil *et al.*, 2007). EGFR and its ligands are highly expressed in tumour cells (Arteaga *et al.*, 2001) and it is suggested that co-expression of EGFR and its ligands is associated with poor survival (Holbro *et al.*, 2004). Furthermore, it is known that the proliferative capacity acquired by overexpression of EGFR may promote tumour progression (Johnson *et al.*, 2004). Other reports indicate that cancer progression through EGFR-mediated signals include mechanisms such as angiogenesis, metastatic spread and apoptosis inhibition (Ciardiello *et al.*, 2001). EGFR and ErbB-2 are frequently overexpressed in several tumours including breast, colorectal, ovarian and non-small cell lung cancer (Roskoski *et al.*, 2004). Additionally, EGF is expressed in human breast carcinomas and human breast lines (Salomon *et al.*, 1995).

Results from Shahbazi *et al.* (2002) showed that a polymorphism in the *EGF* gene results in a nucleotide A to G substitution at position 61.[14] According to these authors and others, G carriers have significantly higher EGF production in peripheral-blood mononuclear cell cultures, glioblastomas, and gliomas cell lines (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a).

To the best of our knowledge, no reports regarding *EGF* polymorphism and breast cancer risk have been published. Therefore, we investigated the association of *EGF* gene A61G polymorphism to breast cancer development.

Material and Methods

Subjects

This case-control study was performed in 883 women including 383 patients with histologically diagnosed breast cancer followed at the Portuguese Institute of Oncology – Porto, and a control group of 500 women without cancer disease history. All participants provided informed consent and the individuals from both groups were from Caucasian ethnicity and were residents in the same geographic area.

Regarding breast cancer patients, the median age at diagnosis was 44 years (mean 46.9, standard deviation 14.1). Concerning histological classification, 89.0% of cases were invasive ductal carcinomas, 4.2% invasive lobular carcinomas and 6.8% consisted of other types (medullary carcinoma; papillary/micropapillary carcinoma; ductal carcinoma in situ; mucinous carcinoma and mixed (ductal and lobular; ductal and papillary)). Patients were

assessed according to the staging system and were followed by the same medical oncologist.

The healthy individuals for the control group were randomly recruited from the Institute's Blood Donor's Bank and had no evidence of neoplastic disease. The median age was 41 years with a mean of 41.7 and 14.7 of standard deviation.

DNA was extracted from peripheral blood samples obtained with a standard venipuncture technique using EDTA-containing tubes, according to previously published studies from our group (Medeiros *et al.*, 2004; Costa *et al.*, 2007b; Costa *et al.*, 2008).

EGF +61 A>G genotype analysis

The *+61 A>G* polymorphism (rs 4444903) was genotyped by the method previously reported by Shahbazi *et al.* (2002) using the PCR-RFLP technique. Amplification was carried out in a 50 µl reaction mixture containing: 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.3 µM of each primer and 1U Taq DNA polymerase. The cycling conditions comprised a hot start at 95ºC for 5 min, followed by 35 amplification cycles at 94ºC for 60s, 55ºC for 60s and 72ºC for 60s, followed by one elongation step at 72ºC for 5 min. A 242 base pair (bp) fragment was amplified using primers: F-5' TGT CAC TAA AGG AAA GGA GGT3' and R-5'TTC ACA GAG TTT AAC AGC CC3'. The A61G variation was identified with the restriction enzyme *Alu*I. 2U restriction enzyme was added to 10 µl PCR products in a final volume of 15 µl. The incubation was performed at 37ºC overnight. The products were separated on 3% agarose gels with 0.5% ethidium bromide and photographed under UV illumination.

After the destruction of the recognition site by the restriction enzyme, the A allele produced 4 fragments: 15, 34, 91 and 102 bp, while the G allele produced 3 fragments: 15, 34 and 193 bp. In the gel only the fragments: 91, 102 and 193 bp were visible.

Statistical analysis

The computer software SPSS for windows (version 15.0) and Epi Info (version 6.04) were used for all statistical analyses. Statistical calculations were performed using χ^2 test being conducted to compare categorical variables, and a 5% level of significance was used in the analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to assess the relationship between the polymorphism and breast cancer. The Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed vs. the expected genotype frequencies.

Furthermore, we considered the waiting time for the disease onset (WTO) as the interval between the time of initial exposure to the risk factor (*+61 A>G*) and the time of disease onset, as reported earlier (Costa *et al.*, 2007b; Costa *et al.*, 2008; Medeiros *et al.*, 2004). Thus, we calculated the cumulative probabilities for having disease by the Kaplan–Meier methodology and the primary analysis of time-to-event end points for WTO performed with the use of a two-sided log-rank test at the 5% level of significance.

Results

EGF genotype frequencies in breast cancer patients and control group are presented in Table 1. Frequencies of the genotypes were 29.8% for AA, 47.2% for AG and 23.0% for

GG in the control group and, 34.7% for AA, 47.0% for AG and 18.3% for GG in the breast cancer group. Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium for control group (p = 0.514). We found that GG carriers present a lower risk for developing breast cancer than AA carriers (OR = 0.68; 95% CI, 0.46-1.01; p = 0.047). Allelic frequencies were also significantly higher for G-allele carriers in controls compared to breast cancer patients (46.6% vs. 41.8%, respectively; p = 0.043) and a lower risk for breast cancer was observed in G allele carriers (OR = 0.82; 95% CI, 0.08-1.00) (Table 1). Furthermore, the analysis for the G allele in cancer susceptibility after stratification for age regarding the median age at diagnosis (44 years) demonstrate no statistically significant difference in the younger group (under 44 years) or in the older group (p=0.637 and p=0.591, respectively). Moreover, no difference were observed after stratification for stage (p=0.462).

Table 1 EGF genotype and allele frequencies in patients with breast cancer and in healthy controls.

	Cases (n=383)		Controls (n=500)		OR	95% CI	p
	n	%	n	%			
Genotypes [a]							
AA	133	34.7	149	29.8	1		
AG	180	47.0	236	47.2	0.85	0.62-1.17	0.310
GG	70	18.3	115	23.0	**0.68**	0.46-1.01	**0.047**
Alleles							
A allele	446	58.2	534	53.4			
G allele	320	41.8	466	46.6	**0.82**	0.08-1.00	**0.043**

[a] Analysis for linear trend according to the presence of null, one, or two G alleles: p = 0.048.

The cumulative probability of having an earlier breast cancer diagnosis (mean WTO), according to the presence or absence of the GG genotype, was statistically significant (55 years for A allele carriers vs. 59 years for non-carriers; log-rank test: p=55 years for A allele carriers vs. 59 years for non-carriers; log-rank test: p=0.041; OR= 1.353; 95% CI, 1.012-1.808) (Fig. 1).

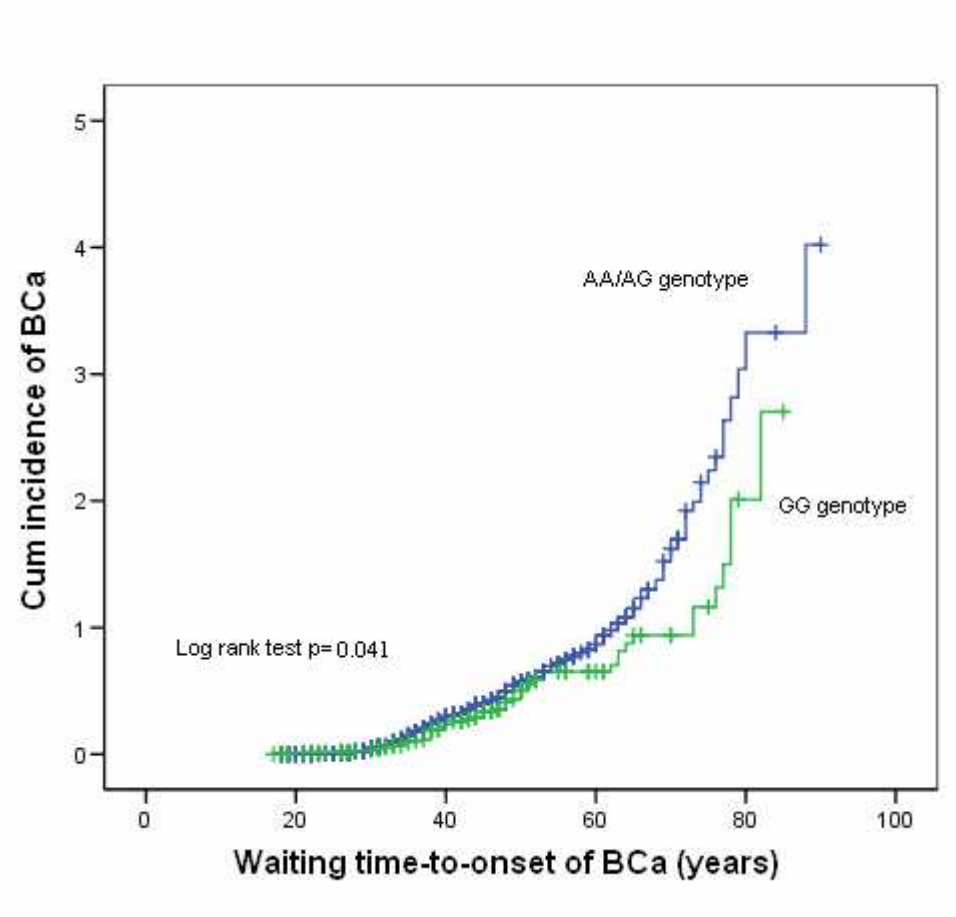

Fig.1 Association between A61G polymorphism and the waiting time to onset of disease (WTO). Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test (p = 0.041).

Discussion

EGF is a growth factor that activates a signalling transduction cascade responsible for activating genes involved in cell progression. The functional polymorphism *+61 A>G* in the *EGF* gene has been associated with increased risk for melanoma, glioma, gastric cancer, among others (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a; Rees *et al.*, 2002; McCarron *et al.*, 2003; Amend *et al.*, 2004; Hamai *et al.*, 2005; Ribeiro *et al.*, 2007), and it has been suggested that GG/AG carriers have higher production of EGF than AA carriers (Shahbazi *et al.*, 2002; Bhowmick *et al.*, 2004; Costa *et al.*, 2007a) although no previous study has been published regarding the association of this polymorphism with breast cancer risk.

Our results demonstrated that *EGF* polymorphism may influence breast cancer risk. We observed that GG carriers have a lower risk for developing this neoplasia (OR=0.68; 95% CI, 0.46-1.01). Furthermore, allelic frequencies distribution suggest a protective role for breast cancer in G alleles carriers (OR = 0.82; 95% CI, 0.08-1.00). Moreover, we demonstrated a later onset of breast cancer in GG carriers (log-rank test: p=0.041). Our results must be observed with caution. In this study mean age of controls is lower than the mean age for the case group and age is a well know risk factor for cancer development. Further studies must include the adjustement of results for risk factors (age, smoking, alcohol drinking habits) and the information regarding survival may be a valuable tool for the understanding of the role of EGF in the follow up of breast cancer.

When compared with other ligands, EGF has distinct biological events and its actions are promoted by "down-regulation" of EGFR that will go to condition the different intracellular pathways activated (Taketani *et al.*, 1983). In fact, a plausible biological explanation for our results may be based upon a recently established mechanism for EGF-activation of EGFR internalization and degradation (Friedman *et al.*, 2005). According to this hypothesis, the EGF/EGFR complex is internalized through endossomal mechanism and as EGF is not sensible to acidic pH, dissociation does not occur, not allowing receptor recycling to the membrane surface (Wells *et al.*, 1999; Singh; *et al.*, 2005). It is interesting to observe that the EGFR is not internalized either if the ligand is TGF-α (Wells *et al.*, 1999; Singh; *et al.*, 2005) or if the ligand is EGF and its receptor are associated with ErbB-2 or ErbB-3 (Lenferink *et al.*, 1998). Furthermore, other studies demonstrate an abrupt decrease at surface expression of EGFR when cell lines are exposed to high levels EGF (Salazar *et al.*, 2002; Friedman *et al.*, 2005). Zhao *et al.* (2006) recently demonstrated that a high concentration of EGF induced loss of adhesion, cell cycle arrest, apoptosis, and inhibition of proliferation in cell lines. Thus, the putative higher EGF production of GG carriers may be consistent with breast cancer protection.

Another possible explanation could be the involvement of EGF in mammalian development (Taketani *et al.*, 1983; Fisher *et al.*, 1990; Spitzer *et al.*, 1995; Rosfjord *et al.*, 1999; Dehnhard *et al.*, 2000), and the ductal and alveolar epithelium stimulation (Fisher *et al.*, 1990; Spitzer *et al.*, 1995). EGF is localized in the inner layers of the terminal end bud and in ductal cell of mammary epithelium (Snedeker *et al.*, 1991). Also, EGF is present in the milk, being secreted by the mammary gland tissue during lactation (Fisher *et al.*, 1990), and with high concentrations being found inclusively after weaning (Fisher *et al.*, 1990; Beardmore *et al.*,1983; Moran *et al.*, 1983; Brown *et al.*, 1989). Interestingly, it was

suggested that breast cancer risk decreases with breast feeding, with having a child under the age of 24 and with an additional pregnancy (Russo *et al.*, 2005).

Results from the present study are consistent with the hypothesis of EGFR internalization in association with higher available EGF levels in breast cancer. A decrease in breast cancer development risk may be explained by internalization of EGF receptor (EGFR) promoted by EGF. Further studies may explore the role of EGF in definition of chemoprevention strategies or its meaning in therapeutic response of breast cancer.

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6.3 Anexo III

Provas do artigo intitulado *"Epidermal growth factor genetic variation and advanced cervical cancer in younger women"*, submetido para publicação na revista *Cancer Genetics and Cytogenetics*.

Epidermal growth factor genetic variation and advanced cervical cancer in younger women

Ana Paula Araújo[a,b], Ricardo Ribeiro[a,c], Deolinda Pereira[d], Daniela Pinto[a,c,e], Raquel Catarino[a,c], Rui Medeiros[a,c,e,f]

[a]Molecular Oncology Group, Portuguese Institute of Oncology - Porto, Portugal

[b]Department of Biology, University of Aveiro, Portugal.

[c]ICBAS, Abel Salazar Biomedical Sciences Institute, University of Porto, Portugal

[d]Medical Oncology Department, Portuguese Institute of Oncology - Porto, Portugal

[e]Virology Department, Portuguese Institute of Oncology - Porto, Portugal

[f]Faculty of Health Sciences of Fernando Pessoa University, Porto, Portugal

Correspondence:

Prof. Rui Medeiros

Molecular Oncology Group

Portuguese Institute of Oncology, Porto Centre

Edifício Laboratórios-PISO 4

Rua. Dr Ant. Bernardino Almeida

4200-072 Porto

Portugal

E-mail: ruimedei@ipoporto.min-saude.pt.

Abstract

Epidermal growth factor (EGF) stimulates cells proliferation through binding to its receptor (EGFR) and the overerexpression of this receptor is associated with a poorer prognosis. The *EGF* gene presents a polymorphism at position 61 (A/G), associated with higher EGF production. We examined the association between this polymorphism and cervical cancer through a case-control study. It was determined by the PCR-RFLP method in 384 women with cervical lesions and 500 controls from Caucasian ethnicity. Regarding patients with cervical cancer, we found an increase in the risk of advanced disease (FIGO stage IIb/IV) in younger G carriers (OR=3,17; 95% CI=1.214-8.257, p=0.016). We may hypothesize the onset of an advanced disease driven selective pressure due to the effect of oncogenic HPV types in a favourable genetic background observed in G carriers women. Present results suggest that EGF functional polymorphism may influence cervical cancer prognosis through EGF/EGFR pathway.

Keywords: EGF, polymorphism, cervical cancer, EGFR

Introduction

EGF is a growth factor discovered by Cohen (1962) [1] from the purified extracts of the salivary gland and then was associated with the function of stimulating the proliferation of epidermal tissues [2], after the binding to epidermal growth factor receptor (EGFR) [3]. Gray et al., 1983 showed that EGF is a polypeptide with 53 amino acids but it is synthesized from a large protein precursor of 1168 amino acids [4], and Morton *et al.* (1986) located the gene in locus 4q 25-27 [5].

It is known that the EGFR is a pleiotropic signaller that activates pathways involved in cell proliferation, migration and differentiation [6]. EGF binds to its receptor and induces a signalling cascade that culminates in cell transcription [7, 8]. This signal transduction also occurs in cancer cells [9] and tumours with overexpression of EGFR are strongly associated with a poor prognosis [10]. Because the EGFR are associated to bad prognosis in tumour progression fate is necessary to implement the new therapeutic targets and network strategies [11, 12]. Cervical cancer is the second most common cancer in women, with 493,000 estimated new cases and 274,000 deaths in the year 2002 [13] and the majority of cervical carcinomas express high levels of the EGFR [14].

Shahbazi *et al.* (2002) identified a functional polymorphism at position 61 of the *EGF* gene, which consists on a substitution of adenine (A) for guanine (G). AA genotype carriers have lower levels of EGF expression than individuals with the GG or AG genotypes [15].

In this case-control study we analysed this polymorphism in association with the risk of developing cervical cancer in a high incidence Caucasian population.

Material and Methods

Subjects

In this case control study, 884 women were genotyped for the *EGF* A61G polymorphism. Three-hundred and eighty four (384) had cervical lesions (that included 233 with invasive cervical carcinoma (ICC)), and 500 had non-cancer controls. The patients were recruited at the Portuguese Institute of Oncology, Porto centre, between 1999 and 2004, according to previously reported studies [16-20]. The median age at diagnosis for women with squamous intraepithelial lesions (SIL) was 35 years and 47 for women with ICC. In the group of patients with ICC 78.4% had squamous histologcal type, 12.4% adenocarcinoma and 3.9% other types. Tumour staging was assessed according to the FIGO staging system and were followed by the same medical oncologist. Individuals from the control group did not report clinical or pathological cervical disease and were recruited from the Institute's Blood Donors Bank, with 41 years of median age. All participants provided informed consent according to the Helsinki Declaration and the individuals from both groups were from Caucasian ethnicity and were residents in the same geographic area.

EGF A61G genotype analysis

Genomic DNA was extracted from 8ml of blood obtained with a standard venipuncture technique using EDTA-containing tubes of which was utilized the white blood cell fraction of each sample, using a standard salting out protocol [21].

The EGF A61G polymorphism was analyzed by PCR-RFLP essay using two primers (F-5' TGT CAC TAA AGG AAA GGA GGT3' and R-5'TTC ACA GAG TTT AAC AGC CC3') as described previously by Shahbazi *et al.* (2002) [15]. The PCR reaction was

carried out in a 50µl mixture containing 1x Taq Buffer, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.3 µM of each primer and 1U Taq DNA polymerase. These reaction mixtures were heated at 95ºC for 5 min; 35 cycles at 94ºC for 60 s, 55ºC for 60 s and 72ºC for 60 s; and a final extension step at 72ºC for 5 min. 10 µl PCR products were digested overnight at 37ºC with 2 U *Alu*I restriction enzyme to a final volume of 15 µl. The restriction fragments were then analyzed by electrophoresis in 3% (w/v) agarose gels stained with 0.5% ethidium bromide and photographed under UV illumination.

Statistical analysis

SPSS for windows (version 15.0) and Epi Info (version 6.04) were used for all statistical analyses. Statistical calculations were performed using χ^2 test in categorical variables. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated to assess the association of the genetic variant with cervical disease and/or cancer. The Hardy-Weinberg equilibrium was tested by a Pearson of goodness fit test to compare the observed vs. the expected genotype frequencies.

Results

The frequencies of EGF genotypes in the cervical lesions group and the control group are exhibited in table 1 and 2. The frequencies of the genotypes were 29.8% for AA and 70.2% for AG/GG in the control group, 28.9% for AA and 71.1% for AG/GG in the entire group with cervical lesions and 27.0% for AA and 73.0% for AG/GG in the group with ICC. Genotype frequencies for this polymorphism were in Hardy-Weinberg equilibrium

for both groups (controls: p = 0,514; cases: p = 0,978). No significant differences were found in subjects who carried G lack of increased risk of developing cervical lesions (p=0.773), and ICC (p=0.443), as shown in Table 1.

Table1: Association of A61G polymorphism with clinicopathological parameters in patients with cervical lesions

	Cases		Controls		OR	95%CI	p
	n	%	n	%			
Cervical lesions							
Genotypes							
AA	111	28.9	149	29.8	1		
AG	189	49.2	236	47.2	1.08	0.78-1.49	0.649
GG	84	21.9	115	23.0	0.98	0.66-1.45	0.918
AG+GG	273	71.1	351	70.2	1.04	0.78-1.40	0.773
ICC							
Genotypes							
AA	63	27.0	149	29.8	1		
AG	117	50.2	236	47.2	1.17	0.80-1.72	0.397
GG	53	22.8	115	23.0	1.09	0.69-1.73	0.700
AG+GG	170	73.0	351	70.2	1.15	0.81-1.62	0.443

When we made adjustments concerning age, histology and staging, we found an increased risk of G carriers genotypes with stage IIb-IV (considered as advanced disease) and younger than 47 years were associated with an advanced cancer (OR, 3.166; 95% CI, 1.214- 8.257; p=0.016) (Table 2). For other characteristics no associations were found (Table 1 and Table 2).

Table2: Association for advanced disease[a)] in G carriers (A61G polymorphism) in women under the age of 47 years

	p	OR	95%CI
Advanced disease[a)]	0.064	1.89	0.96-3.70
Advanced disease[a)] with age ≤ 47	**0,016**	**3.17**	**1.21-8.26**
Advanced disease[a)] with age > 47	0,520	0.69	0.22-2.15

[a)]Advanced disease: FIGO stage IIb/IV (reference localized disease: stage I/IIa)

Discussion

Cervical cancer has a straight reasoning linking the disease with the genital human papillomavirus (HPV), considering that the high-risk HPV (hr-HPV) genotypes are detected in almost 100% of all cervical cancers [23]. Cervical cancer develops through a continuum of progression of the different degrees with cytological abnormalities culminating in carcinoma. However, the presence hr-HPV infections are necessary, but not sufficient, to be the cause of this carcinoma [24]. The persistence of HPV infection is mediated by several factors - oral contraceptive use, parity, other STIs (Sexually Transmitted Infections), smoking, nutrition and host genetics [24]. Currently it was shown that genetic variability might play a significant role in cervical cancer predisposition [16-19, 25-28].

The objective present study was to examine the relationship between a functional EGF polymorphism (A61G) and cervical cancer. Results showed an increased risk for advanced disease in younger women (OR=3.17). This result may be explained through the higher production of EGF in G carries, which might increase cell proliferation signalling. Other

studies in melanoma, glioma, gastric cancer, observed that this polymorphism was associated with an increased risk for cancer development [15, 19, 30-35]. Additionally, a previous study failed to demonstrate cervical cancer risk association with EGF genotypes in East Asian individuals [36]. To the best of our knowledge, we present the first study in Caucasian women regarding the cervical cancer risk and EGF polymorphism. A study by Kang *et al.* (2007) of Korean patients revealed increased tendency of lymph node in G carriers [36]. Our results are consistent with this report however we observed that younger women simultaneously G carriers had increased risk of advanced cervical cancer (Table 2).

It is known that the EGFR pathways contribute to cell proliferation, apoptosis, angiogenesis and metastatic spread. All these processes are involved in cancer development and progression [37]. It has been suggested that EGFR overexpression might drive to the acquisition of proliferative capacity and tumour progression by tumour cells [38]. Moreover, earlier clinical reports suggest that coexpression of EGFR and its ligands (including EGF) are associated with poor survival of patients [12] and in cervical dysplasias 85-100% can be detected EGFR expression [14]. Also in cervical cancer, it was showed that EGF signalling is enhanced in E5-expressing cells [39-41]. Straight et al. (1993) demonstrated that the levels of EGFR are higher in E5-expressing keratinocytes cells because exist an apparent delay in the internalization and degradation of this receptor, and a greater number of receptors return to the cell surface than control keratinocytes, as well as a greater magnitude of ligand-stimulated phosphorylation of the EGFR tyrosine residue [39]. Recently, a report from Chiang *et al.* (2007) demonstrated that EGF is involved in the activation of cell invasion through upregulation or stimulation of NHE I

(Na+/H+ exchanger isoform I), in cervical cancer cells [42].This facts can explain our association between EGF and cervical cancer.

Concerning our observation of an increased advanced cancer the risk associated with women younger than 47 years we may suggest an association with the prevalence of HPV infection (low-risk or high-risk) to be relatively higher in this group when compared with older women [43-45]. We may hypothesize that it may exists an advanced disease driven selective pressure due to the effect of oncogenic HPV types in a favourable genetic background observed in G carriers women.

With concerning to association found for advanced stages, Gaffeney *et al.* 2003 demonstrated that patients with EGFR expression have less disease-free survival and overall survival, and concluded that the lower disease-free survival was higher as FIGO stages [47]. According to our results we suggest that the EGF/EGFR pathway only significantly impacts the risk for advanced ICC in younger women (< 47 years).

In conclusion the HPV is the first established risk factor in cervical cancer, although EGF A61G polymorphism may also influence this neoplasia. This fact only happens in women younger than 47 years and leads to advanced stages. This can be explained by younger women to have more HPV infection prevalence, and on the other hand, the action of EGF and its signalling are associated with poor prognosis in cervical cancer patients.

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